A New Strategy for Enhancing the Stability of Lyophilized Protein: The Effect of the Reconstitution Medium on Keratinocyte Growth Factor

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Purpose. Protein stabilization during lyophilization has previously focused on optimization of the formulation as well as the freezing and dehydration process parameters. However, the effect of the reconstitution medium has been largely neglected. We have investigated its effect on aggregate formation using recombinant keratinocyte growth factor (KGF).

Methods. The protein was lyophilized under suboptimal conditions to induce aggregation and precipitation upon reconstitution with water. A series of additives were examined by UV spectrophotometry and size exclusion chromatography (SEC-HPLC) for their effects on decreasing the degree of KGF aggregation and precipitation by the increase in recovery of soluble monomer.

Results. Several additives resulted in a significant reduction of aggregation, including sulfated polysaccharides, surfactants, polyphosphates, and amino acids. A similar effect was achieved by adjusting the ionic strength of the reconstitution medium. SEC-HPLC indicated that the amount of soluble monomer was also increased by these additives suggesting that the recovery of the soluble protein correlates with the native, monomeric protein.

Conclusions. These results suggest that optimization of reconstitution conditions will be a useful methodology for increasing the recovery of soluble, active proteins and that for KGF, the recovery of the soluble protein correlates with the native, monomeric form.

KEY WORDS: proteins; aggregation; reconstitution; lyophilization; additives; stability.

INTRODUCTION

Formulation of proteins is a challenge, since proteins are relatively unstable in the aqueous state. Proteins can undergo chemical and physical degradation resulting in a loss of biological activity during processing and storage (1). Protein degradation is typically associated with irreversible unfolding, aggregation, and chemical modifications. In cases where the stability of an aqueous preparation is inadequate, the process of freeze-drying (lyophilization) is employed to stabilize proteins for long-term storage. Protein stability following lyophilizaton is a function of environmental factors, including temperature, humidity, pH, ionic strength, and solvent medium composition. However, in spite of an increased shelf-life after freeze-drying, some proteins lose biological activity during the process. Typical practices to improve protein stability include the addition of stabilizers such as carbohydrates and amino acids to the protein solution prior to freeze-drying (2–4).

Reconstitution of the lyophilized protein is necessary before administration. Standard practice is to add back a volume of pure water although dilute solutions of antibacterial agents are sometimes used in products for parental administration (5). Although there has been considerable research in developing methods to maintain the stability of lyophilized proteins, it has been limited to the optimization of the freeze-drying formulation and process. Little is known about protein structure and stability during the reconstitution process. Early studies showed profound changes in protein conformation, dynamics, and other properties during the transition from a dry solid protein to the fully rehydrated one (6). However, these studies have focused upon the slow sequential rehydration via the introduction of air of controlled relative humidity. Reconstitution of lyophilized proteins is typically performed by rapidly replacing the initial volume. This is expected to differ from sequential rehydration because both equilibrium and kinetic processes are involved. In this study, the capacity of specific additives and ionic strength to decrease aggregation of the lyophilized protein during the reconstitution step has been explored using a recombinant keratinocyte growth factor (KGF). The understanding of the reconstitution process should lead to the development of rational stabilization strategies and hence to improvement in the stability of pharmaceutical proteins.

MATERIALS AND METHODS

Materials

A human recombinant KGF produced using *escherichia coli* expression was used. Sulfated β-cyclodextrin was purchased from American Maize (Hammond, Indiana). Sodium polyphosphates were purchased from Aldrich (Milwaukee, Wisconsin). All other sulfated polysaccharides, amino acids, salts, and surfactants as well as ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, Missouri). All references in the text to solute concentration are on a weight to volume (w/v) percentage basis unless specifically stated otherwise.

Preparation, Filling, and Freeze-Drying

KGF (wild type or the oxidized wild type) at a concentration of 3–4 mg/ml was thoroughly dialyzed against 10 mM potassium phosphate buffer, pH 7.5 using Spectro/por 7 cellulose dialysis tubing (Spectrum) at 4°C. Protein concentration was determined spectrophotometrically using the extinction coefficient of 1.03 at 280 nm. Sufficient buffer was added to the stock solution to adjust the protein concentration to 0.40–0.65 mg/ml in the desired pH buffers. The solutions were passed through a 0.22 μm-pore size filter (Millex GV filter, Millipore Corp., Bedford, Massachusetts) and filled in 1 ml aliquots into sterile, 3 ml glass vials (Baxter Scientific Product). Butyl rubber freeze-dry stoppers (1-mm diameter, West Co., Phoenixville, Pennsylvania) were inserted halfway into the vials and the vials were transferred to the freeze-dryer shelves (FTS Systems, Stoneridge, New

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York), which had been pre-cooled to −50°C. The temperature of the samples was maintained at −50°C for 2 hours. Primary drying was carried out at a vacuum of less than 100 mTorr, a condenser temperature below −60°C, and a shelf temperature at −45°C for 12 hours followed by a shelf temperature at −10°C for 10 hours. Secondary drying was at +20°C for 10 hours. At the end of the freeze-dry cycle, all the vials were stoppered under vacuum. Aluminum overseals were crimped into place prior to storage. The freeze-dried samples were stored at 45°C, and removed at appropriate intervals for reconstitution and aggregation measurements.

Aggregation Measurements

The amount of protein aggregation was monitored by obtaining the absorbance at 280 nm using a Beckman DU 650 UV-visible spectrophotometer. Each lyophilized sample was reconstituted in 1 ml of water or additive solutions followed by centrifugation (14000 g, 20 minutes at 4°C) to remove insoluble protein. The degree of aggregation (expressed as percentage of aggregate) was calculated by comparing the absorbance of the supernatant to the absorbance of the pre-lyophilized sample. Any absorbance interference by the additives was corrected for by using the additive buffer as the reference. No light scattering corrections were necessary after the centrifugation, which removed most or all of the aggregated proteins. The concentration of the soluble protein (mg/ml) was calculated by using the extinction coefficient of 1.03 at 280 nm. The stabilization ratio is defined here as the extent of soluble protein when the lyophilized protein was reconstituted with an additive solution normalized to that reconstituted in pure water. Thus, a value of 1.0 indicates that the additive has no effect on aggregation. A value greater than 1.0 indicates enhanced stabilization, while a value less than 1.0 means destabilization by the additive.

High-Performance Size-Exclusion Chromatography (HP-SEC)/Light Scattering

HP-SEC of protein was carried out at 25°C on a Pharmacia Superdex 75 column (10 × 30 cm I.D., Pharmacia Biotech, Inc., Alameda, California). The quantitation of KGF monomeric peak was accomplished using Hewlett-Packard 1090 series II with a diode array detector and HP 1047A refractive index detector. PBS buffer plus 1 M NaCl, pH 7.0 (isocratic conditions) was used as the mobile phase at a flow rate of 0.5 ml/min. This high salt elution buffer was required to dissociate additives, e.g., heparin, which potentially bind to the proteins and to reduce non-specific interactions of the proteins with the column matrix. Elution of protein was followed by absorbance at 280 nm and by refractive index. The protein sample (100 μl) was injected onto the system by an auto sampler. A standard calibration curve was generated using the known concentrations of pre-lyophilized KGF samples. The concentration of soluble protein (mg/ml) after reconstitution was determined based on the standard calibration curve.

The molecular weight of the eluted peaks from HP-SEC was determined by using an on-line light scattering/chromatography system. The eluent was monitored by three detectors in series: a Knauer A293 absorbance monitor at 280 nm (Wissenschaftl. Geratebau Dr. Ing.H. Knauer GmbH, Germany), a Wyatt mini-Dawn laser light scattering detector (Wyatt Technology Corporation, Santa Barbara, California) and a refractive index detector operating at 650 nm (Polymer Laboratories PL-RI, Amherst, Massachusetts). The molecular weight of the elution peak was calculated from the ratio of the light scattering to refractive index signals as described by Takagi (7). Elution buffer was 1.0 M NaCl in PBS. The system was operated at a flow rate of 0.5 ml/min. Ribonuclease, ovalbumin, and bovine serum albumin (monomer) were used to determine instrument constants.

Biological Activity Assay

The mitogenic activity of KGF was determined using Balb/MK cell lines. Balb/MK cells are plated at fifty thousand cells per well, incubated for 48 hours, treated with growth factor, and processed as described by Rubin *et al.* (8). The data were expressed as a [^{3}H]-thymidine uptake over background level.

RESULTS AND DISCUSSION

Keratinocyte growth factor (KGF) is a recently identified member of the FGF family (FGF-7) having a molecular weight of 18.5 kDa and consisting 163 amino acid (9). KGF was used as a model protein in this study due to its propensity to aggregate during long time storage at elevated temperature. From amino acid sequence, the tertiary structure of KGF may resemble to that of acidic or basic fibroblast growth factor (FGF) (10).

In the present investigation, several potential excipients with differing physicochemical properties were used to evaluate their ability to prevent KGF aggregation and precipitation. Purified KGF in aqueous solution has been shown to aggregate rapidly upon storage (11). Addition of highly sulfated polysaccharides such as heparin increase the stability of the protein (11). Heparin also enhances the stability of KGF during lyophilization (unpublished results). It is interesting to see if heparin has any effect on the recovery of soluble KGF upon reconstitution. The experimental approach was to expose the lyophilized proteins to a more stressful storage condition, i.e., high temperature, and compare the loss of soluble protein after reconstitution in the presence or absence of the additives.

Figure 1 shows that the extent of aggregation increases with storage time and that aggregation was greater after reconstitution in water than in either heparin or sucrose octasulfate (SOS). Wide type KGF was formulated with 10 mM potassium phosphate, pH 7.0 buffer. When the lyophilized KGF sample was rehydrated with water containing either heparin or SOS, aggregation was 10 to 15% that rehydrated with water. The effect of heparin concentration on the extent of aggregation upon reconstitution is shown in Figure 2. The results were obtained with a different preparation from that used to generate the results in Figure 1. KGF in the oxidized form was used and formulated with 5% mannitol in 10 mM potassium phosphate buffer (pH 7.0). The preparation used in Figure 2 and the following studies are less prone to aggregation. Heparin at a concentration as low as 0.002% was sufficient to reduce aggregation. Further reduction in aggre-

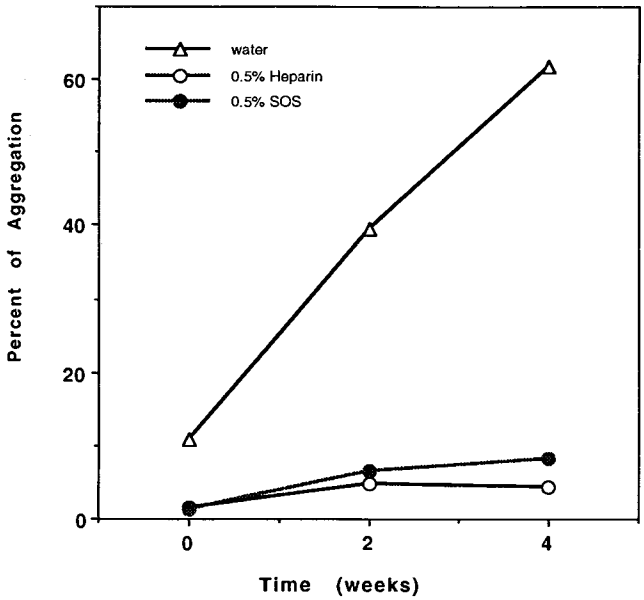

Fig. 1. Amount of aggregation for lyophilized KGF upon reconstitution with water, 0.05% heparin (16K) and SOS. The lyophilized samples contained 10 mM potassium phosphate buffer, pH 7.0.

gation was observed with increasing heparin concentrations up to 0.05%. Above 0.05%, no additional reduction of aggregation was observed, indicating that there is some fraction of the aggregation event which was not impeded by heparin under the conditions tested.

Next, a wide variety of sulfated polysaccharides and highly charged compounds were screened. Some have been shown to be effective stabilizers against heat-induced aggregation when formulated with other heparin-binding proteins (12). A summary of these reconstitution experiments in terms of the extent of aggregation is illustrated in Table I; The sulfated compounds varied significantly in their ability to stabilize KGF upon reconstitution. For example, heparin, sulfated β- cyclodextrin, fucoidan, chondroitin sulfate B and dextran sulfate, are more effective in reducing aggregation than pentosan polysulfate, chondroitin sulfate C, which in turn are more effective than chondroitin sulfate A and myo-inositol hexasulfate. Other small, highly charged and phosphorylated molecules are observed to significantly decrease the extent of aggregation. Among the compounds tested, sodium polyphosphate is the most effective agent having a stabilization ratio of 1.77. The pH of the reconstituted samples remained essentially unchanged compared to the pre-lyophilized sample.

Amino acids varied significantly in their ability to reduce aggregation upon reconstitution, as shown in Table II. Among the monomers, for example, sodium aspartate is much more effective than glutamate. Among the homopolymers and random copolymers of amino acids, poly-L-glutamic acid, for example, is more effective than poly-L-lysine and poly (lys, ala). Surfactants (Tween-20), cyclic oligosaccharides (hydroxyl β- cyclodextrin) and metal chelators (EDTA) also have the capacity to reduce KGF aggregation during rehydration. In contrast, no significant reduction in aggregation was observed when the lyophilized samples were reconstituted with water containing the surfactants pluronic or N-octylglucoside.

The influence of ionic strength on the extent of KGF aggregation accompanying rehydration was also examined. Figures 3A and 3B show the degree of KGF aggregation when rehydrated in the presence of various amounts of NaCl, and $(NH_4)_2SO_4$, respectively, versus control (water alone). Measurements were taken immediately after lyophilization or after 3 weeks of storage at 45°C. The resulting aggregation measured immediately after lyophilization, com-

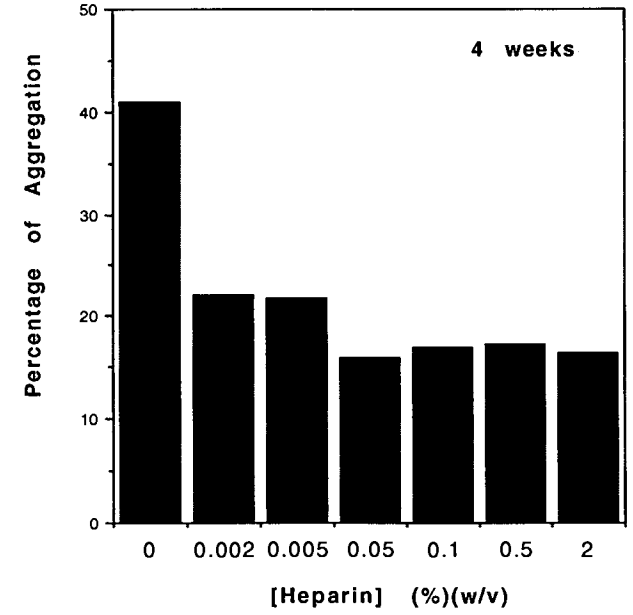

Fig. 2. Effects of 16 K heparin concentration on KGF aggregation after 4 weeks storage at 45°C and reconstitution with 1 ml heparin solution. The lyophilized samples contained 5% mannitol in 10 mM potassium phosphate buffer, pH 7.0.

Table I. Amount of Soluble KGF After Lyophilization and Storage at 45°C and Reconstitution by Polyanions[a]

Reconstitution medium	Stability ratio	
	t = 0	t = 2 weeks (45°C)
Control	1	1
0.5% Heparin	1.07	1.87
0.5% Dextran Sulfate	1.07	1.74
0.5% Fucoidan	1.08	1.71
0.05% Pentosan polysulfate	1.07	1.67
0.1% Chondroitin Sulfate A	1.07	1.24
0.5% Chondroitin Sulfate B	1.06	1.73
0.1% Chondroitin Sulfate C	1.06	1.65
0.05% Myo-inositol Sulfate	1.07	1.26
0.5% Sulfated β-cyclodextrin	1.09	1.79
0.05% Polyphosphoric Acid	1.19	1.77
0.5% Na-pyrophosphate	1.12	1.64
0.5% Di-Na Di-H pyrophosphate	1.01	1.16
0.5% Na-tripolyphophate	1.09	1.46
0.05% Tetrapolyphosphate	1.17	1.42
0.1% Phosvitin	1.09	1.32

[a] The lyophilized KGF samples contained 5% mannitol in 10 mM potassium phosphate buffer, pH 7.0 (see Materials and Methods).

Table II. Amount of Soluble KGF After Two Weeks at 45°C Storage and Reconstitution by Nonspecific Agents[a]

Reconstitution medium	Stability ratio
Control	1.0
10 mM Histidine	1.18
60 mM Histidine	1.06
66 mM Glycine	1.08
660 mM Glycine	1.52
3 mM Sodium aspartate	1.36
15 mM Sodium aspartate	1.74
30 mM Glutamate	1.17
27 mM Lysine hydrochloride	1.11
0.5% Poly-L-glutamic acid	1.30
0.5% Poly-L-lysine	1.11
0.1% Poly(acrylic acid)	1.13
0.05% Poly(lys, ala)	1.04
0.5% PEG 300	1.17
2.0% PEG 300	1.19
0.05% Pluronic	1.03
0.5% Tween-20	1.42
0.05% N-Octylglucoside	1.09
1.0% Hydroxypropyl β-cyclodextrin	1.25
10 mM Imidazole (HCl)	1.23
50 mM Imidazole (HCl)	1.50
0.05% EDTA	1.20

[a] The lyophilized KGF samples contained 5% mannitol in 10 mM potassium phosphate buffer, pH 7.0 (see Materials and Methods).

pared to that rehydrated with water only, was reduced by 30% and 50% in the presence of 200 mM NaCl and $(NH_4)_2SO_4$, respectively. Aggregation measured after 3 weeks of storage was suppressed by NaCl at high concentration, but not by $(NH_4)_2SO_4$. In fact, the degree of aggregation was enhanced with increasing concentration of $(NH_4)_2SO_4$ (Figure 3B). Thus, it appears that the degree of aggregation is influenced by the ionic strength and that the effect of the ionic strength on aggregation is salt specific. It is not clear why the effects of ammonium sulfate are opposite depending on the storage time and hence the extent of aggregation. Similar reduction of aggregation was observed when lyophilized KGF samples stored at 45°C for 3 weeks were reconstituted with sodium citrate at moderate and higher concentrations (50 mM to 0.5 M) (not shown). Other agents such as imidazole at moderate concentration (50 mM) and glycine (660 mM) were also effective in reducing aggregation accompanying reconstitution, as shown in Table II.

These results demonstrate that the amount of soluble protein can be greatly increased using reconstitution medium containing additives. In order to examine the entity of the soluble protein, HP-SEC was carried out. The results for the lyophilized KGF stored at 45°C for 2 and 10 weeks and reconstituted with the sulfated polysaccharides are summarized in Table III. The reconstituted KGF samples eluted at the elution position of the aqueous, native KGF sample (23 minutes). The amount of native protein calculated from the peak area decreased for the sample reconstituted with water only, while the samples reconstituted in water containing the indicated additives showed an increased recovery of the native, monomeric protein. The molecular weight of the peak was determined by light scattering technique as described by

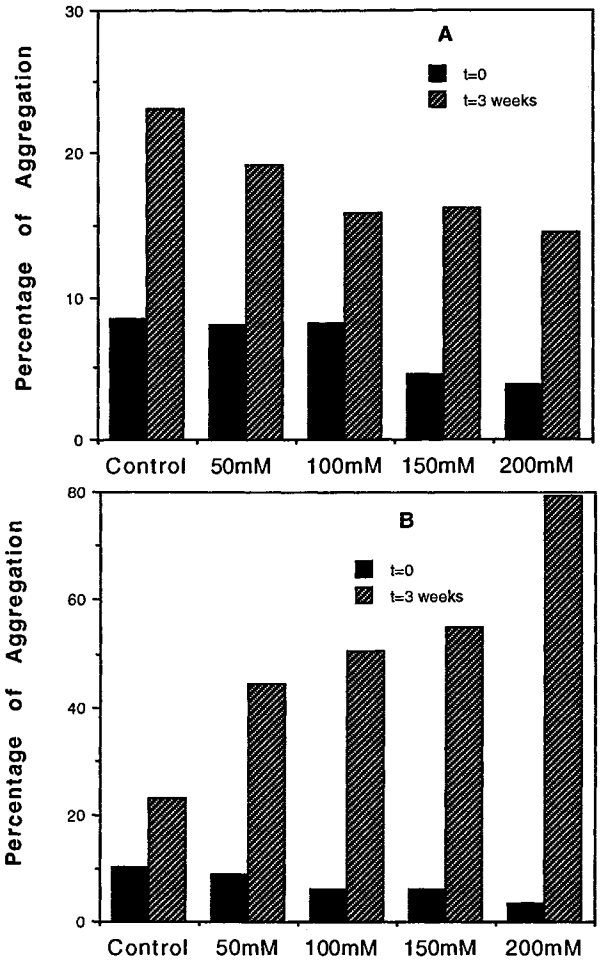

Fig. 3. Effects of salt concentration on KGF aggregation stored at 45°C and reconstituted with 1 ml NaCl (A) or $(NH_4)_2SO_4$ (B) solution. The lyophilized KGF samples contained 4.5% mannitol, 0.5% sucrose in 10 mM potassium phosphate buffer, pH 7.0 upon reconstitution with 1 ml reconstitution medium.

Takagi (7) and was calculated to be 18.5 K, corresponding to the monomeric form of KGF (Figure 4). Similar results are observed with compounds such as poly-L-glutamic acid or phosvitin (Table IV). Peaks at the high molecular weight region of the HP-SEC elution profile is not observed, which is evidence that the soluble protein corresponds to the native, monomeric form of the protein. Thus, we have shown in this

Table III. HP-SEC Peak Area of Soluble KGF After 45°C Storage and Reconstitution Sample[a]

	2 weeks		10 weeks	
Reconstitution medium	Peak area	Soluble protein (mg/mL)	Peak area	Soluble protein (mg/mL)
Control	2771	0.48	2528	0.44
0.5% Heparin	3200	0.56	2960	0.52
0.5% Sulfated β-cyclodextrin	2959	0.51	2678	0.47
0.05% Na polyphosphate	3245	0.56	2953	0.51

[a] The lyophilized KGF (oxidized form) samples contained 10 mM potassium phosphate, pH 7.5 (see Materials and Methods).

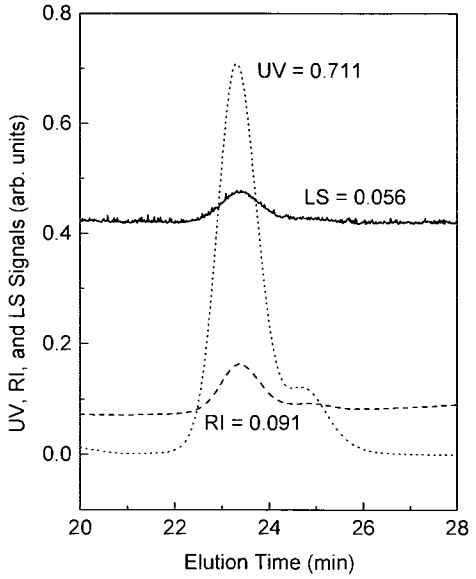

Fig. 4. Typical HP-SEC chromatogram of the KGF reconstitution samples with light-scattering, refractive index, and absorbance detection (see materials and methods). The lyophilized KGF (oxidized form) samples contained 10 mM potassium phosphate buffer, pH 7.0. UV: ultra violet; RI: refractive index; LS: light scattering.

study that the recovery of soluble protein can be monitored by protein concentration measurements of centrifuged supernatant by UV absorbance and by peak area of HP-SEC. Similar elution position for the starting material and reconstituted samples for these protein preparations suggests that they have similar conformations. Since aggregation and unfolding increase the hydrodynamic radius, elution position is a good indicator of native conformation using this technique.

We next investigated aggregation from two reconstitution conditions. In one case, a volume (0.5 ml) of water containing an additive was first added to the lyophilized sample followed by an equal volume (0.5 ml) of water. In the second case, water was added first followed by an addition of equal volume of water containing an additive. Using the same techniques as mentioned above, the amount of soluble protein for each of the reconstitution conditions was determined. As Table IV indicates, samples that were reconstituted with additive solution first (Case a) in general showed a higher soluble protein recovery as determined by peak intensity and absorbance than when reconstituted with water first (Case b). The apparent higher concentration of soluble protein observed by UV may be due to contaminants present in the sample. They can be separated out by HP-SEC. Addition of the additives following reconstitution (i.e. as a 2nd step) had little or no effect on reducing protein aggregation. Thus, the observed reduction in aggregation by solution containing stabilizers is due to the presence of the additives in the reconstitution medium. Furthermore, the fact that a higher degree of aggregation is observed with the latter method indicates that aggregation formed after reconstitution is not reversible by the additives.

A parallel experiment in which an *in vitro* cell mitogenesis assay was used to assess the aggregation of KGF was performed and the results are summarized in Table V. The results indicate that the activity of KGF increases as expected when KGF was reconstituted with a solution containing 0.05% sodium polyphosphate at 10 and 20 weeks of storage, or 0.5% sulfated β-cyclodextrin at 20 weeks of storage. Evidence from HP-SEC and UV showed that the monomeric form of KGF is increased in the presence of the additives. On the contrary, although the levels of the monomeric form of KGF increase when reconstituted with 0.5% heparin as an additive (Table IV), an unexpected decrease in KGF activity is observed. Studies by others showed a similar observation in which the mitogenic activities of KGF were found to be strongly inhibited by heparin (13). Thus, lower molecular weight polyanions such as sulfated β- cyclodextran and dextran sulfate may be used to substitute for heparin in the reconstitution medium.

Our study has demonstrated that aggregation of KGF can be greatly reduced during the reconstitution step. Previous studies have demonstrated that the optimization of lyophilization formulation and protocol can result in sub-

Table IV. Amount of Soluble KGF After 20 Weeks at 45°C Storage and Reconstitution Under two Different Conditions

Case #	Reconstitution medium	Protein concentration (mg/ml) HP-SEC	Protein concentration (mg/ml) UV (280 nm)
	Control	0.41	0.44
1[a]	0.5% Heparin/H_2O	0.45	0.50
1[b]	H_2O/0.5% Heparin	0.42	0.45
2[a]	0.05% Na polyphosphate/H_2O	0.47	0.60
2[b]	H_2O/0.05% Na polyphosphate	0.42	0.46
3[a]	0.5% Sulfated β-cyclodextrin/H_2O	0.45	0.51
3[b]	H_2O/0.5% Sulfated β-cyclodextrin	0.39	0.47
	0.5% Poly-L-glutamic acid[c]	0.46	0.54
	0.1% Phosvitin[c]	0.44	0.52

[a] The lyophilized samples were first reconstituted with a volume (0.5 ml) of water containing an additive followed by equal volume (0.5 ml) of water.

[b] The lyophilized samples were first reconstituted with water followed by equal volume of water containing the additives.

[c] The lyophilized samples were reconstituted with 1 ml reconstitution solution. All lyophilized KGF (oxidized form) samples contained 10 mM potassium phosphate, pH 7.0.

Table V. Biological Activity of Lyophilized KGF Samples Under Different Reconstitution Conditions at 45°C Expressed as Ratio of the Control (Water) Sample[a]

Case #	Reconstitution medium	t = 10 weeks Ratio	t = 20 weeks Ratio
1[a]	0.5% heparin/H_2O	0.93	0.43
1[b]	H_2O/0.5% heparin	0.81	0.40
2[a]	0.05% Na polyphosphate/H_2O	1.30	1.60
2[b]	H_2O/0.05% Na polyphosphate	1.14	1.43
3[a]	0.5% sulfated β-cyclodextrin/H_2O	1.07	1.58
3[b]	H_2O/0.5% sulfated β-cyclodextrin	0.88	0.95
	Control	1.00	1.00

[a] (See Table IV).

stantially improved recovery and stability of freeze-dried proteins (14–16). Freeze-drying is commonly the final step in a sequence of carefully controlled processes for the production of valuable pharmaceutical proteins. It is generally assumed that lyophilized proteins remain structurally and functionally intact after reconstitution as compared to the pre-reconstituted state. Little effort has been made towards optimization of the reconstitution conditions for protein stability.

An alternative to rehydration is to freeze dry proteins in the presence of additives. These may not always be practical. Some additives may not be stable when they were lyophilized. In addition, lyophilizing formulations with certain salts, or surfactants may result in decreased protein stability (17).

The mechanism of the stabilization effect during the reconstitution process, however, is still uncertain. More detailed studies are required to further elucidate the mechanisms involved in aggregation during rehydration. One final point worth mentioning concerns interpretation of previous results of studies on lyophilization and stability of proteins. The present study has demonstrated quite convincingly that the reconstitution step is an important determinant in the cumulative stability of lyophilized proteins. However, prior studies, by us and others, have essentially ignored this important step. As a result, data from these studies have been interpreted in terms of either the lyophilized formulation or process and have ignored the effects of the reconstitution on protein stability are a separate process that needs to be independently assessed. Thus, previous studies of lyophilized protein stability may have to be reevaluated in light of the present results.

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