The Effect of the Reconstitution Medium on Aggregation of Lyophilized Recombinant Interleukin-2 and Ribonuclease A

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INTRODUCTION

Proteins are marginally stable and prone to any perturbation including freezing and drying, resulting in loss of structure and biological activity after reconstitution (1, 2). Stabilizers are often included in protein solutions prior to lyophilization to enhance the recovery of native, active proteins. Previous studies have focused on optimization of the type and concentration of the stabilizer for a particular protein. However, it is not necessarily true that proteins remain structurally and biologically intact after reconstitution. In a recent paper, we have demonstrated that a recombinant human keratinocyte growth factor (KGF) aggregates and loses biological activity when the lyophilized protein was reconstituted with water (3). The degree of aggregation increases as a function of storage time. Inclusion of additives in the reconstitution medium such as polysaccharides, polyphosphates, and amino acids reduces aggregation. To our knowledge, the stabilization of a protein by these additives upon reconstitution of the lyophilized protein is the first observation of such action, and hence, it is interesting to see if such reduction in aggregation and enhanced recovery of native, active protein are in fact a universal phenomenon. In this study, a therapeutically important protein, interleukin-2 (IL-2), and a well-characterized protein, ribonuclease A (RNase A), were used as model proteins for testing various additives in their ability to reduce aggregation upon lyophilization and reconstitution.

MATERIALS AND METHODS

Materials

Human IL-2 used in this study is a recombinant protein with a mutation of Cys125 to Ala and was produced using *Escherichia coli* expression, refereed to as IL-2. A bovine pancreatic RNase A (type X-A) was obtained from Sigma (St. Louis, MO). Sulfated β-cyclodextrin was purchased from American Maize (Hammond, IN). Sodium polyphosphates were purchased from Aldrich (Milwaukee, WI). All other sulfated polysaccharides, amino acids, salts, surfactants, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma. Ribonucleic acid (RNA) (type 1 from yeast) was from Boehringer (Indianapolis, IN). All references in the text to solute concentration are on a weight to volume (w/v) percentage basis unless specifically stated otherwise.

Table I. Amount of Soluble IL-2 After Two Weeks of Storage at 45°C upon Reconstitution

Reconstitution Medium	Stability Ratio
Control	1.0
0.5% Heparin	2.8
0.5% Heparin + 0.05% EDTA	3.41
0.5% Dextran Sulfate	2.91
0.5% Sucrose Octasulfate	0.60
1.0% Sucrose Octasulfate	0.17
0.5% Chondroitin Sulfate A	0.70
0.1% Chondroitin Sulfate A	0.95
0.5% Chondroitin Sulfate B	1.21
0.1% Chondroitin Sulfate C	1.47
0.5% Chondroitin Sulfate C	0.78
0.05% Myo-Inositol Hexasulfate	0.85
0.5% Fucoidan	0.88
0.5% Sulfated β-Cyclodextrin	1.21
0.05% Sulfated β-Cyclodextrin	1.00
0.5% L-Lysine hydrochloride	1.31
0.5% Glutamic Acid	1.17
100mM Glycine	1.52
50mM Glycine	1.15
0.5% Poly-L-lysine	2.78
0.5% Poly-L-glutamic Acid	2.57
0.05% Poly(lys, ala)	1.90
0.5% Poly(lys, ala)	0.80
0.05% Na Polyphosphate	0.79
0.05% Na-Tripolyphosphate	0.97
0.05% Di-Na Di-H pyrophosphate	0.93
0.2% Na Tetrapyrophosphate	0.82
1.0% Pluronic	0.65
0.5% Pluronic	0.85
0.1% Tween-20	1.51
0.5% Tween-20	2.07
1.0% N-Octylglucoside	0.50
0.01% N-Octylglucoside	0.89
1.0% 2-Hydroxylpropyl-β-cyclodextrin	1.46
0.5% 2-Hydroxylpropyl-β-cyclodextrin	1.56
0.5% Phosvitin	0.85

Note: All IL-2 samples contain 5% mannitol (see Materials and Methods).

Preparation, Filling, and Freeze-drying

IL-2 at a concentration of 8 mg/ml was extensively dialyzed against 10 mM MES, pH 4.0 overnight at 4°C using Spectro/por 7 cellulose dialysis tubing (Spectrum) at 4°C. IL-2 solutions with pHs other than 4.0 were prepared by a sufficient number of buffer changes at desired pHs several hours before lyophilization. RNase A was stored at −20°C at a concentration of 3–4 mg/mL in 0.2 M sodium phosphate buffer (pH 6.4).

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Following thawing, the RNase A solution was dialyzed against the buffer at pH 10. Lyophilized RNase A has been shown to be very stable at neutral pH. However, aggregates of RNase A were observed at extreme pHs upon storage at 45°C (4). Thus, pH 10 was chosen in this study to shorten the time required to observe aggregation.

Protein concentrations were determined spectrophotometrically using the extinction coefficient of 0.88 for IL-2 and 0.67 for RNase A at 280 nm. Sufficient buffer was added to the stock solution to adjust the protein concentration (0.8 mg/mL, IL-2; 1.5 mg/mL, RNase A) in the desired pH buffers. The solutions were passed through a 0.22-μm-pore size filter (Millex GV filter, Millipore Corp., Bedford, MA) 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, PA) were inserted halfway into the vials and the vials were transferred to the freeze-dryer shelves (FTS Systems, Stoneridge, NY), 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. Primary drying was continued at a shelf temperature of −10°C for 10 hours. This was followed by secondary drying 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 indicated times for reconstitution and aggregation measurements.

Table II. Remaining Activity of Freeze-Dried RNase A Under Different Storage Conditions upon Reconstitution with Water or Additive Solutions Expressed as a Percent of Control (Prelyophilized) Sample

Reconstitution Medium	pH 10.0 Activity Remaining (%) t = 45°C, 3 weeks
Control	61.9
0.05% Na polyphosphate	96.5
0.05% heparin	60.2
0.5% sulfated β-cyclodextrin	77.0

Aggregation Measurements

The amount of IL-2 aggregation was monitored by obtaining the absorbance at 280 nm using a Beckman DU 650 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 stabilization ratio is defined here as the extent of soluble protein when the lyophilized protein was reconstituted with an additive solution normalized to the extent of soluble protein 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.

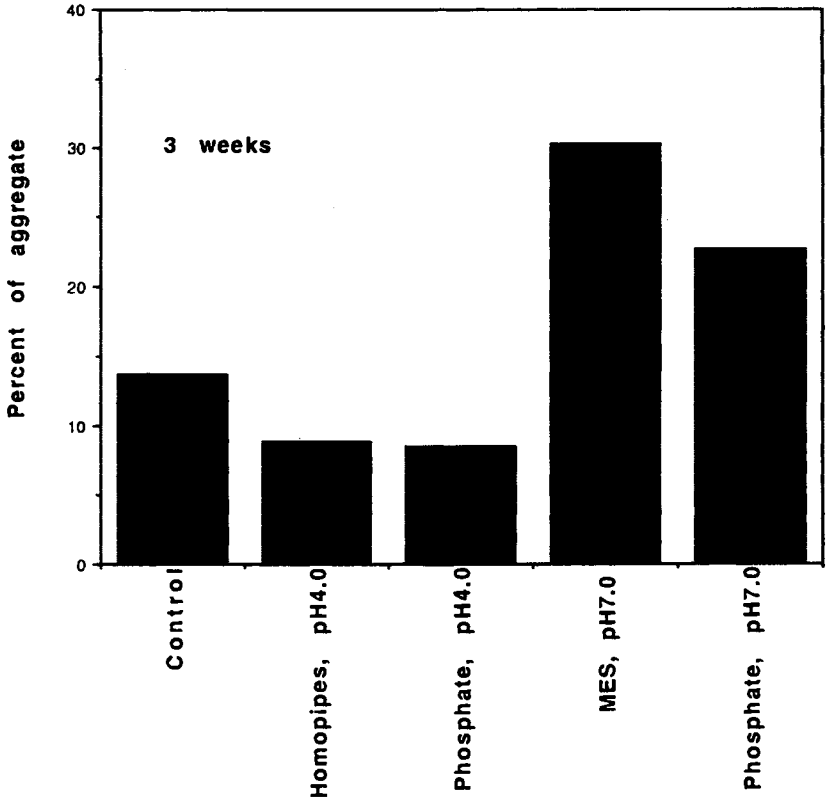

Fig. 1. Effects of pH on aggregation of a lyophilized IL-2 stored at 45°C for three weeks upon reconstitution with buffers. The lyophilized IL-2 samples contain 5% mannitol upon reconstitution with 1 mL volume.

High Performance-size Exclusion Chromatography (HP-SEC)

HP-SEC of RNase A was carried out at 25°C on a Pharmacia Superdex 75 column (10 × 30 cm I.D., Pharmacia Biotech, Inc., Alameda, Ca). 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. Elution of protein was followed by absorbance at 280 nm. The protein sample (100 μL) was injected onto the system by an auto sampler.

Ribonuclease Activity

The specific activity of RNase A was determined as described by Kunitz (5). Lyophilized samples were reconstituted with water or solutions containing the additives 10 minutes before the start of the assay. RNase A specific activities were expressed as a percent of the pre-lyophilized sample at pH 6.4.

RESULTS AND DISCUSSION

A naturally occurring human IL-2 contains a free cysteine at position 125. Substitution of this residue with Ala yields IL-2 analog (Ala-125), which was found to have biological activities slightly higher than those of the native form (6). IL-2 is a fairly hydrophobic protein and is prone to aggregation upon lyophilization and storage at neutral pH (7).

A variety of compounds were tested for their capacity to reduce IL-2 aggregation upon reconstitution. The results are summarized in Table I. Similar to the results observed for KGF, IL-2 aggregation was significantly suppressed when the lyophilized samples were reconstituted with the solution containing heparin, or sulfated polysaccharides such as dextran sulfate, chondroitin sulfate C, and amino acids such as poly-L-lysine or poly-L-glutamic acid. Heparin, dextran sulfate, poly-L-lysine and poly-L-glutamic acid all showed high stabilization ratios, suggesting that poly-ions with high charge density are effective stabilizers. Unlike KGF, aggregation was not suppressed by 0.5% sodium sucrose octasulfate (SOS). At 1% SOS, aggregation was even enhanced. A detrimental effect was observed with several other additives including chondroitin sulfate A, chondroitin sulfate C and poly (lys, ala) at higher concentrations. The polyphosphates, which were effective in reducing aggregation for KGF and RNase A, were detrimental to IL-2.

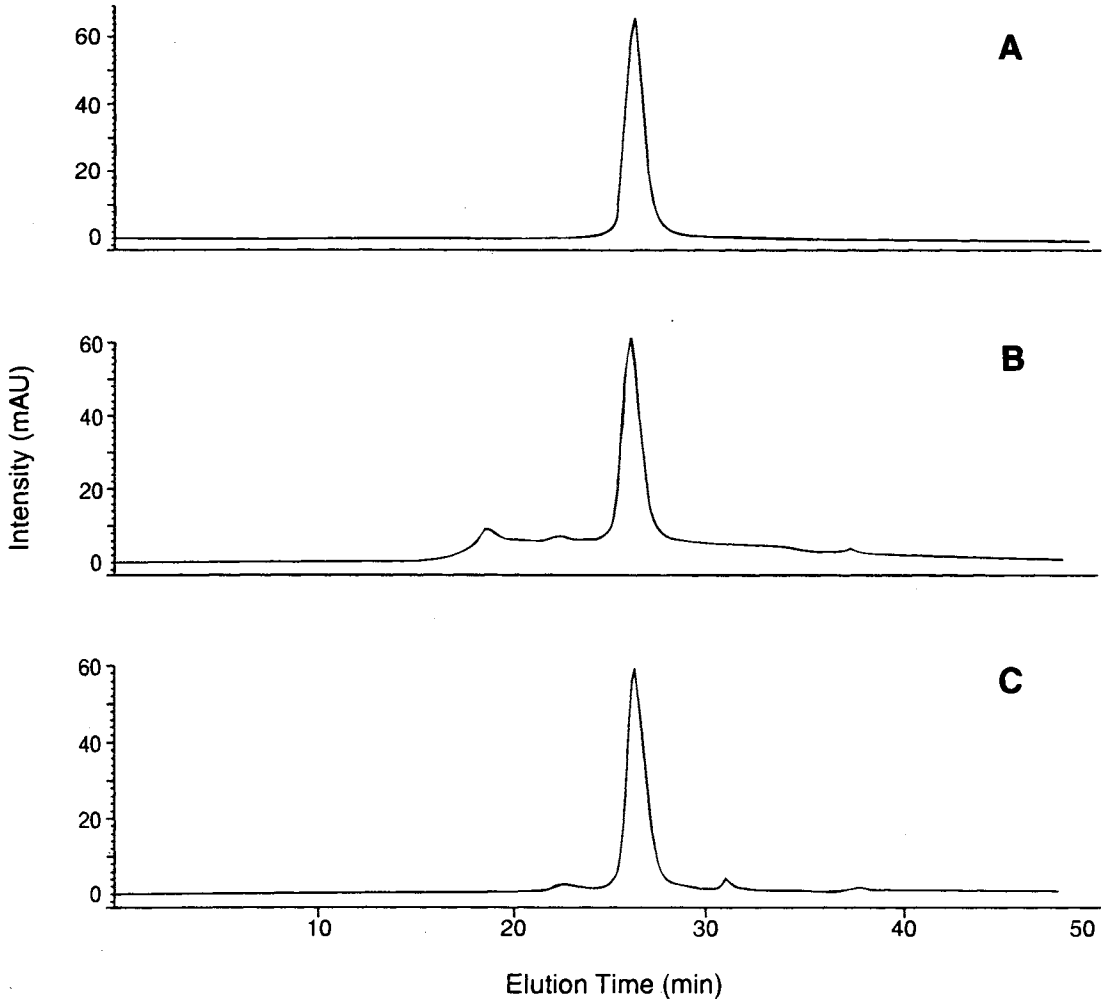

Fig. 2. High-performance size-exclusion chromatograms of ribonuclease A (RNase A) solution. (A) before freeze-drying, (B) freeze-dried and reconstituted with water, (C) freeze-dried and reconstituted with 0.05% sodium phosphate.

Previous studies showed that IL-2 is significantly more stable when lyophilized from a pH 4 solution than when lyophilized from a neutral pH solution (8). It is of interest to determine if all or part of the pH effect observed for IL-2 is manifested during the reconstitution step. For these studies, IL-2 (0.8 mg/ml) was lyophilized from 1 mM MES buffer (pH 7.0) to reduce buffer effects. The lyophilized samples were then reconstituted with 10 mM buffers at two different pHs (4 and 7). Results are shown in Figure 1. The samples rehydrated with water showed less aggregation than those rehydrated with the pH 7.0 buffer. This may be due to a lower ionic strength of pure water than the 10 mM buffer. The extent of aggregate formation was further reduced by rehydration with the pH 4 buffer. The type of buffer appears to have little effect. Thus, it appears that the pH during rehydration has a strong influence on the aggregation of IL-2.

The rehydration study was extended to RNase A, the structure of which has been extensively investigated (9–12). Its enzymatic activity makes this protein attractive to examine the additive effect on the recovery of native protein upon lyophilization and reconstitution.

It has been shown that RNase A aggregates when stored in the freeze-dried form at increased temperature (13, 14). Lyophilized RNase A after storage at 45°C is stable at neutral pH but less stable at 10 (4). RNase A samples lyophilized from pH 10, immediately followed by reconstitution, showed no loss in biological activity, consistent with the results of Crestfield et al. (15). A loss of activity was observed when RNase A was reconstituted after storage at an elevated temperature (45°C) for three weeks. The resulting reduction in activity was due to significantly increased levels of protein aggregation.

The effects of some agents on the residual activity after reconstitution are shown in Table II. For pH 10 formulation, RNase A that was reconstituted with water retained 62% of its original activity. However, if reconstituted with 0.05% sodium polyphosphate, or 0.5% sulfated β-cyclodextrin, the residual activity increased up to 97% or 77%, respectively. No increase in activity recovery was observed when reconstituted with 0.5% heparin, which has been shown to be effective in reducing aggregation for both KGF and IL-2. It is well known that RNase has a high affinity for phosphates (16–18).

The reconstituted solution of lyophilized RNase A showed no apparent aggregation and precipitation and hence was studied by HP-SEC for soluble aggregates. A typical chromatogram of HP-SEC is shown in Figure 2. The lyophilized RNase A showed elution of larger molecular species when reconstituted with water, whereas the prelyophilized material essentially displayed a single peak. These larger molecular species were reduced significantly when reconstituted with water containing 0.05% polyphosphate, consistent with the enzyme assay.

CONCLUSION

Both IL-2 and RNase A showed significant aggregation upon storage at 45°C, when pure water was used for reconstitution. The extent of aggregation was greatly reduced by including various additives such as heparin or phosphates in the reconstitution medium. Together with the results of the previous paper, these results demonstrate that optimization of reconstitution medium is an alternative way to increase the recovery of the lyophilized proteins.

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