Thermal Stability of Low Molecular Weight Urokinase During Heat Treatment. II. Effect of Polymeric Additives

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Turbidimetric or light scattering assays can be used to determine the extent of aggregation in protein formulations. Using low molecular weight urokinase (LMW-UK) as a model protein, the effect of polymeric additives on heat-induced aggregation was evaluated. Previous work has shown that under 60°C heat treatment, LMW-UK initially denatures and the unfolded protein associates to form soluble aggregates. Eventually, these aggregates associate to form a precipitate. The effects of polymers on the initial aggregation phase was examined. Hydroxyethyl (heta) starch, polyethylene glycol 4000, and gelatin were found to be effective, concentration-dependent inhibitors of aggregation, whereas polyvinylpyrrolidone (PVP) and polyethylene glycol 300 were ineffective. Overall, the effect of polymeric additives on the stability of thermally-stressed LMW-UK can be accounted for by preferential exclusion of the solute from the surface of the protein.

KEY WORDS: protein stability; aggregation; turbidimetry; urokinase; formulation; additives, polymeric.

INTRODUCTION

Therapeutic proteins that are derived from blood or circulatory organs have the potential for viral contamination. Consequently, viral inactivation is a necessary step in the processing of these proteins. For example, urinary plasminogen activator (urokinase or UK) is a thrombolytic agent used to dissolve pulmonary emboli, and is produced commercially in kidney cell culture. Plasminogen activators are involved in the conversion of plasminogen to plasmin, and, as such, represent an important target for thrombolytic therapy. Both high molecular weight (HMW-UK, molecular weight ~55 kD) and low molecular weight (LMW-UK, molecular weight ~33 kD) forms of UK have been isolated. Because the LMW-UK produced for pharmaceutical applications (Abbokinase, Abbott Laboratories) is derived from a circulatory organ, there exists a possibility of viral contamination.

Typically, viruses are destroyed by a heat treatment cycle, presumably by thermal denaturation of the coat proteins of the viruses. The heat treatment employed for Abbokinase consists of heating the protein in aqueous solution at 60°C for ten hours. This procedure has been approved by the Food and Drug Administration for inactivation of viruses in blood products. Although effective in removing viral contamination, the heat treatment also leads to a loss of approximately 20% of the active LMW-UK. Much of the degraded protein precipitates and is removed by filtration.

Our previous work has demonstrated that the mechanism of inactivation of heat-treated LMW-UK involves aggregation of either partially or fully denatured protein (1). Circular dichroism spectroscopic studies show that the lag time observed in the turbidimetry measurements correlates with loss of secondary structure due to unfolding (1). The denatured protein rapidly associates to form soluble aggregates which scatter light. Eventually, the soluble aggregates associate and precipitate from solution. The optimal solution conditions (pH, ionic strength, protein concentration) for retarding this process have been identified (1). Thus, the next step in the assessment of formulation variables is to evaluate various excipients for their ability to retard either the initial unfolding or the subsequent aggregation processes.

The ability of turbidimetric assays to assess the extent of aggregation of proteins is well established (2–6). In this study, the effects of polymeric additives on the thermally-induced aggregation of a model protein, LMW-UK, is evaluated. In addition, the usefulness of light scattering methods in evaluating protein formulations is demonstrated.

MATERIALS AND METHODS

Materials

All LMW-UK was from commercial production and was obtained from the main fraction from the final purification column, but prior to any heat treatment (courtesy of Don Eisenhauer of CAPD at Abbott Laboratories). The stock solution of LMW-UK had an activity of 154,000 U/ml and had a protein concentration of 1.3 mg/ml. It also contained 2.0% (w/v) NaCl.

The buffers used were made from stock solutions of 0.2 M sodium monobasic phosphate (Mallinckrodt, lot# 7892 KDPV) and sodium dibasic phosphate heptahydrate (Mallinckrodt, lot# 7914 KEKC). Mixtures of these two solutions were prepared in order to achieve the desired pH following the method of Gomori (7).

Gelatins (type A, lot no. 37f05501 and type B, lot no. 97f0748) were obtained from Sigma, hydroxyethyl starch (molecular weight of 200 kD) was a gift from DuPont Pharmaceuticals (lot no. W87-1-1C), polyvinylpyrrolidone (PVP, lot no. 45C-0035, mol. weight 40,000) was obtained from Sigma, polyethylene glycol (PEG) 300 (lot no. 438 507) and PEG 400 (lot no. 651618) was obtained from J. T. Baker, PEG 4000 came from City Chemical (lot no. 98E125), and glycerol was obtained from Mallinkrodt (lot no. 5092 KHBJ). All chemicals were used without further purification.

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Equipment

A Cary 219 and a Beckman DU-64 UV-visible spectrophotometers were used for all spectroscopic experiments described in this report. Each was attached to a Lauda RM-6 circulating water bath in order to maintain a constant sample temperature of 60°C (bath temperature of 66.2°C). The cuvettes used were obtained from Hellma, having a pathlength of 10 mm and a sample volume of 1.0 to 1.5 ml.

Methods

The spectrophotometer and water bath were allowed to equilibrate at 60°C for a minimum of one hour prior to use. As described previously, the aggregation of LMW-UK can be monitored by following apparent absorption increases in the near ultraviolet or visible regions of the spectrum (1). In order to maximize sensitivity, the absorbance was typically measured at 300 nm. The time course for the reaction was monitored for periods up to one hour, beginning with insertion of the sample into the thermostated cell holder.

For a typical sample, the sample volume was 1.2 ml and the activity of LMW-UK was 38,500 U/ml (~0.33 mg/ml). As noted previously (1), higher protein concentrations lead to faster aggregation rates. The concentration dependence was verified on samples ranging from 19,250 to 105,500 U/ml. However, the lower concentrations provided adequate sensitivity and minimal sample requirements. For LMW-UK, the concentrations were similar (~0.3 mg/ml) to all other samples.

Statistical significance was determined by a standard t test for two different distributions. The number of samples ranged from three to ten for each formulation.

RESULTS

Previously, it was demonstrated that heat treatment of LMW-UK leads to rapid unfolding, followed by association to form soluble aggregates (1), which is but one method for inducing aggregation (8). Eventually, these aggregates form in sufficient concentrations that they associate and precipitate from solution. Solution conditions which reduce the loss of LMW-UK during heat treatment have been identified (1). A relatively narrow pH range, from approximately pH 6.0 to pH 7.0, is available within which maximal activity of the enzyme can be maintained during heat treatment. It has also been shown that ionic strength effects are important and that a minimal amount of either buffer and/or salt (>250 mOsM at pH 6.5) is needed to achieve maximal stability.

It is well known that a variety of compounds can be employed to stabilize protein formulations (9–12). This work focuses on the ability of polymeric excipients to stabilize heat-induced aggregation of a protein. The denaturation of LMW-UK appears to occur either for only a labile fraction of the protein molecules or involves only localized unfolding. In either case, stabilization will increase the denaturation temperature (T_m), and will retard the subsequent aggregation step. Therefore, it was felt that monitoring aggregation was a reliable indicator of the stability-enhancing ability of a particular excipient.

Since the pH range for maintaining maximal activity during heat treatment was between 6 and 7, all samples of LMW-UK were buffered at pH 6.5 using 20 mM phosphate buffer. A brief evaluation of buffer concentrations versus LMW-UK (results not shown) demonstrated that a phosphate buffer concentration of 20 mM was sufficient to minimize aggregation during heat treatment, consistent with the threshold effects of ionic strength that were observed previously (1).

Turbidimetric measurements can be made on conventional ultraviolet spectrophotometers (1,3,5,6), usually at wavelengths where the protein does not absorb. Initial experiments were conducted to determine the sample-to-sample variability of this assay. Each control sample contained 38,500 U/ml of LMW-UK in 20 mM phosphate buffer (pH 6.5). The reproducibility for these samples was relatively good. Figure 1 summarizes the results from eight independent runs. The data were averaged and standard deviations calculated. The relative standard deviation (r.s.d.) at 20 minutes was 6.4% and rose to 10.4% at 30 minutes. Over the course of this study, the overall r.s.d. at 30 minutes was found to be 5.9%, provided temperature was controlled within 0.2°C. Variability (i.e., r.s.d.) for solutions containing high concentrations of polymeric additives could rise to as much as 25–30% (see below), but was frequently much lower. Overall, the procedure is fairly reproducible and comparisons can be made with confidence, provided the differences are greater than 10–20%.

Effect of Gelatin on the Thermal Stability of LMW-UK. Gelatin is obtained by acid or base treatment of animal skins, bones, and cartilage. Acid treatment leads to type A gelatin (gelatin A) while base treatment produces gelatin B. Both types of gelatin were evaluated for their effect on LMW-UK aggregation. Gelatin was added to the formulation in concentrations of 1.0%, 0.5%, and 0.1% (w/v). For samples containing gelatin B at a concentration of 0.1%, there seems to be a small (but not significant) advantage over the control sample, whereas concentrations of 0.5% and 1.0% produce a significant lowering of the amount of aggregation (Figure 2). Addition of 0.1% of gelatin A actually produces an increase

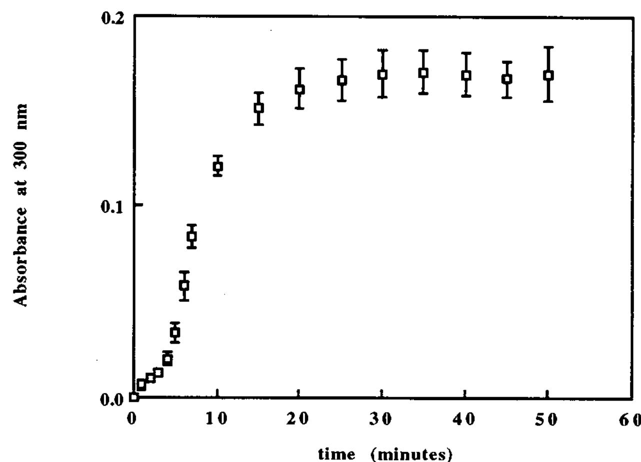

Fig. 1. Average aggregation for LMW-UK (n = 8) heat treated at 60°C as determined by light scattering at 300 nm for samples containing 20 mM phosphate buffer. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

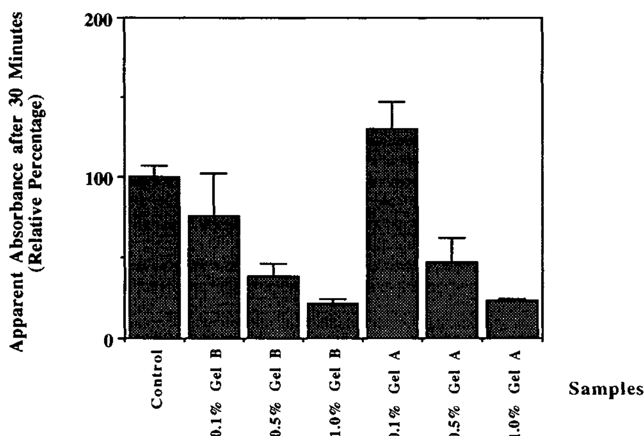

Fig. 2. Extent of aggregation in samples containing gelatin as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

in aggregation. Increasing the concentration of gelatin A to 0.5% and 1.0% provides thermal protection similar to that of type B gelatin (see Figure 2). For formulations containing 1.0% gelatin, the degree of stabilization approaches that of highly purified LMW-UK (see below). Overall, gelatin appears to be the most effective additive at reducing the aggregate formation in heat-treated LMW-UK.

The variability of LMW-UK samples containing gelatin was examined. In general, the r.s.d. in the assay for solutions containing only 0.1% gelatin was greater than that found for buffered LMW-UK alone (cf. Figure 2). However, as gelatin concentrations increased, the reproducibility increased markedly, with 1% gelatin samples exhibiting an r.s.d. of 1–3%. Consequently, gelatin was found to decrease LMW-UK aggregation significantly only at concentrations equal to or greater than 0.5% ($p < 0.005$).

Effect of PVP on the Thermal Stability of LMW-UK. Polyvinylpyrrolidone (PVP) is another polymer reported to stabilize proteins (9). Addition of PVP to LMW-UK solutions at concentrations up to 10% had either no effect or a detrimental effect on heat-induced aggregation of LMW-UK (Figure 3).

Effects on Glycerol on the Thermal Stability of LMW-UK. Another widely used class of excipients in protein formulation are polyols, including simple sugars, oligosaccharides, celluloses, starches, glycerol, polymeric alcohols, and cyclodextrins (8–14). The effect of sugars on stabilizing LMW-UK during heat treatment will be described in a subsequent publication. However, glycerol, as a model polyol, was examined for its effects on LMW-UK, and found to have a significant stabilizing effect (Figure 4).

Another aspect of additive stabilization which may prove to be of importance is the ability for two additives to act independently and stabilize a protein. This may also help distinguish between specific and nonspecific interactions of like solutes with the protein surface. For example, when glycerol is used in conjunction with PEG 4000, the effect is approximately additive for the two excipients (Figure 5).

Effect of Hydroxyethyl (heta) Starch on the Thermal Stability of LMW-UK. Starches represent acceptable pharmaceutical excipients. Heta starch has been developed and approved as a plasma expander. At low concentrations (2% w/v), heta starch has little or no effect on LMW-UK aggregation (Figure 6). However, at higher concentrations, the thermal protection afforded by heta starch is significant. It appears concentrations in excess of 10% are needed to provide stabilization comparable to 1% gelatin. The stabilization of LMW-UK was found to be significant for all concentrations 5% or greater ($p < 0.01$). Even at 2% (w/v) heta starch, the decrease in aggregation was significant to a p value of 0.05.

Effect of PEGs on the Thermal Stability of LMW-UK. Polyethylene glycols (PEGs) are polyethers (with terminal alcohol groups) which display both hydrophobic and hydrophilic character. The ability of PEGs to function as thermoprotective agents is dependent on the temperature, the hydrophobicity of the protein, and the molecular weight of the PEG (15–17). Two different PEGs were examined, low molecular weight (300 and 400 daltons) and high molecular weight (4000 daltons).

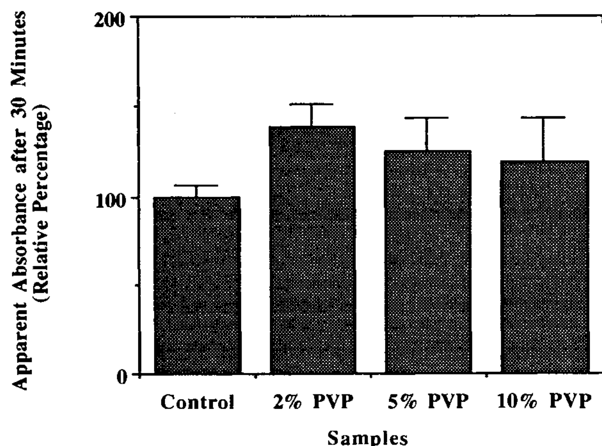

Fig. 3. Extent of aggregation in samples containing PVP as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

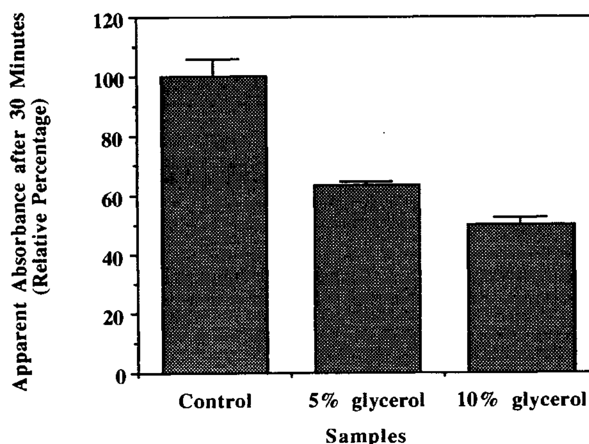

Fig. 4. Extent of aggregation in samples containing glycerol as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

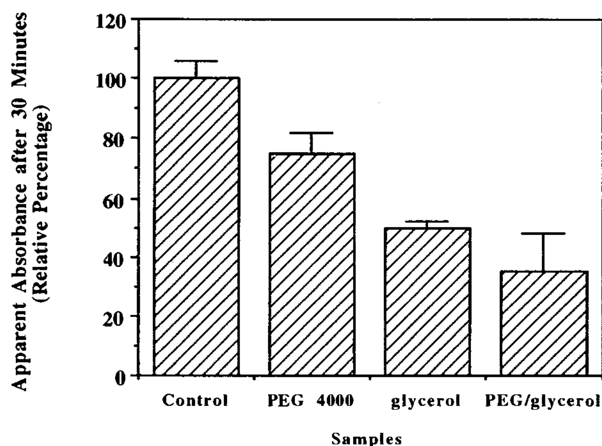

Fig. 5. Extent of aggregation in samples containing combinations of 10% (v/v) PEG 4000 and glycerol as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

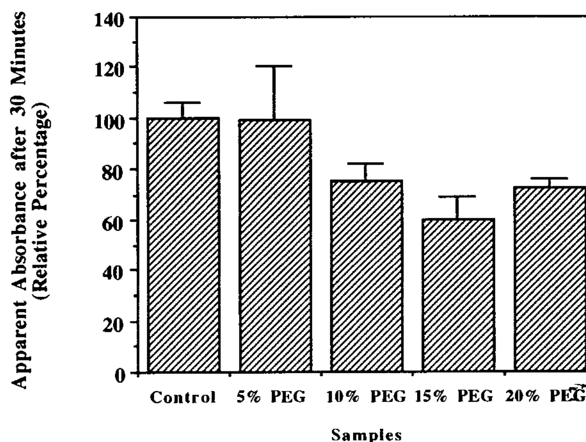

Fig. 7. Extent of aggregation in samples containing PEG 4000 as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

At concentrations less than 5%, PEG 4000 does not significantly decrease aggregation of heat-treated LMW-UK. However, at higher concentrations some thermoprotection is afforded (Figure 7). The basis for stabilization of proteins by PEG appears to be via increasing the denaturation temperature (T_m) through preferential solute interactions with the protein (15–17). This preferential solute interaction is size dependent (16,17), with larger PEGs displaying greater degrees of stabilization (Figure 8). The effects of PEG 300 and PEG 400 were similar, so the only data for PEG 300 are presented.

Formulation of LMW-UK with both glycerol and PEG 4000 provides additional stabilization, but not in any synergistic manner (Figure 5). These results would be consistent with independent effects on the preferential solute interaction of LMW-UK, and are consistent with a lack of specific binding by either solute. PEG has been shown to exhibit preferential binding at high temperature with some proteins (17). Therefore, glycerol was chosen as the most likely to disrupt PEG binding. However, since the effects are roughly additive, no evidence for preferential binding was observed.

DISCUSSION

The basis for the thermoprotective effect of polymers on heat-treated LMW-UK is not known, but most likely arises from stabilizing LMW-UK relative to thermal denaturation. Previous studies on the thermal inactivation of LMW-UK indicated that the initial step was unfolding, which was followed by aggregation (1). Therefore, an increase in temperature required to unfold a protein would lead to less aggregation.

It has been shown that preferential exclusion of a solute from the surface of a protein can increase the thermodynamic stability of the protein (15–19). Timasheff, Arakawa and colleagues observed that there is a deficiency of stabilizing solutes (sugars and PEG) in the immediate vicinity of the protein relative to the bulk solution, and that the protein

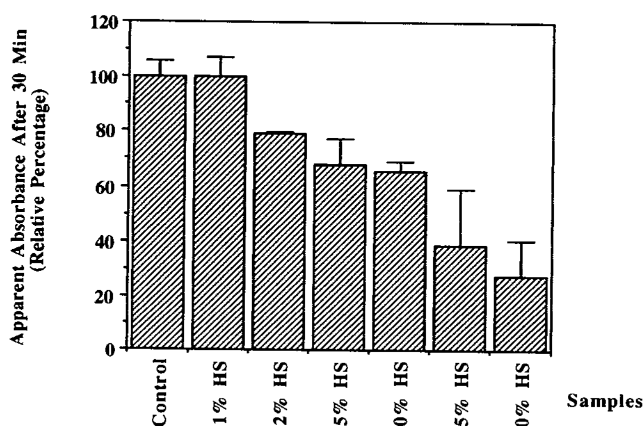

Fig. 6. Extent of aggregation in samples containing heta starch as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

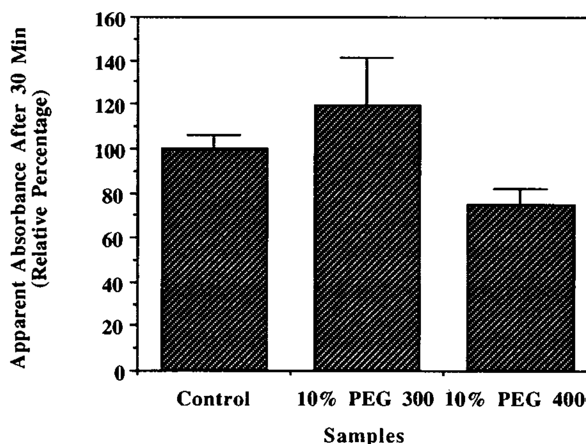

Fig. 8. Extent of aggregation in samples containing 10% (w/v) PEG 300 and PEG 4000 as determined by the apparent absorbance at 300 nm after 30 minutes of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38,500 U/ml.

is preferentially hydrated. The presence of the solute created a thermodynamically unfavorable situation, because the chemical potential of both protein and solute are increased. Stabilization of the protein arises because denaturation would lead to increased surface area and increased contact between the protein and solvent, subsequently augmenting this thermodynamically unfavorable event. The details of this mechanism have been reviewed by Timasheff and Arakawa (18).

The failure of PVP to diminish aggregation may be due to the hydrophobic character of the polymer increasing as the temperature increases, even though similar polymers (e.g., PEG) have been shown to be preferentially excluded at 20°C (15–17). Arakawa and co-workers (12) have proposed that as the temperature is increased, the hydrophobic character of the solute predominates and solutes bind preferentially to proteins. Since the denatured state has more hydrophobic binding sites than the native state, the degree of preferential binding will be greater for the unfolded protein. Thus, there will be a greater decrease in chemical potential of the unfolded state relative to the folded conformation, which leads to stabilization. Also, specific binding of PVP to LMW-UK at high concentrations cannot be ruled out.

The results with PEG are consistent with earlier findings that the preferential exclusion of PEG is dependent on its molecular weight, as the exclusion arises from steric hindrance (15). Consequently, the lower the molecular weight of the compound, the more it should form hydrophobic interactions with the protein (as with PVP). This trend is very clear from the destabilizing effect of 10% PEG 300 versus a stabilizing effect for PEG 4000.

As for the glycerol/PEG mixtures, we were attempting to evaluate whether protein stabilization by two solutes would be directly additive or non-additive. In fact, the results were additive, consistent with the conclusion that the preferential interaction of the two solutes with the protein was not altered by the presence of the other solute. Since the solutes appear to act independently, it may be possible to take advantage of additive stabilization by two or more solutes, when the degree of stability obtained from a single solute is insufficient.

Finally, these data suggest that gelatin is either excluded to a higher degree than the other polymers or exhibits nonideality to a greater extent (19), as both effects lead to stabilization via preferential solute interactions. In either case, gelatin increases the thermal stability of LMW-UK to a greater degree than the other polymers in this study.

Again, it should be noted that the only mechanism of protein stabilization which is consistent with the results of this study is Timasheff's thermodynamic preferential exclusion mechanism. The protein does not appear to be stabilized by specific binding nor by viscosity changes. There are no data to support the notion that viscosity ever plays a role in stabilizing protein structures. In fact, the data in this paper are inconsistent with a viscosity-based mechanism, in that high concentrations of heta starch (with a high viscosity) is much less effective at diminishing aggregation than low concentrations of gelatin (with a much lower viscosity).

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