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Arginine and lysine reduce the high viscosity of serum albumin solutions for pharmaceutical injection

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Therapeutic protein solutions for subcutaneous injection must be very highly concentrated, which increases their viscosity through protein–protein interactions. However, maintaining a solution viscosity below 50 cP is important for the preparation and injection of therapeutic protein solutions. In this study, we examined the effect of various amino acids on the solution viscosity of very highly concentrated bovine serum albumin (BSA) and human serum albumin (HSA) at a physiological pH. Among the amino acids tested, L-arginine hydrochloride (ArgHCl) and L-lysine hydrochloride (LysHCl) (50–200 mM) successfully reduced the viscosity of both BSA and HSA solutions; guanidine hydrochloride (GdnHCl), NaCl, and other sodium salts were equally as effective, indicating the electrostatic shielding effect of these additives. Fourier transform infrared spectroscopy showed that BSA is in its native state even in the presence of ArgHCl, LysHCl, and NaCl at high protein concentrations. These results indicate that weakened protein–protein interactions play a key role in reducing solution viscosity. ArgHCl and LysHCl, which are also non-toxic compounds, will be used as additives to reduce the solution viscosity of concentrated therapeutic proteins.

[**Key words:** Viscosity; Arginine; Lysine; Serum albumin; Protein–protein interaction]

Therapeutic proteins account for an increasingly large proportion of pharmaceutical drugs in recent years. These proteins are normally administered by subcutaneous injection (1). When a high dose of therapeutic proteins is required, the protein solution must often be very highly concentrated to reduce the injection volume for patient convenience. However, such a high protein concentration frequently leads to high viscosity, posing considerable challenges for both processing and injection (2–4). For example, subcutaneous injection is generally performed with solutions under 50 cP to reduce the time required for injection (5). Therefore, maintaining a solution viscosity below 50 cP is a primary goal when developing high concentration formulations of therapeutic proteins.

The viscosity of a protein solution is caused by noncovalent protein–protein interactions such as electrostatic attraction or repulsion, leading to the formation of a transient three-dimensional network of protein (6). There has been considerable effort directed toward lowering the viscosity of concentrated protein solutions through the application of various solution additives (7–11). Among them, inorganic salts (12,13) and hydrophobic salts (5,14) have been shown to effectively reduce the viscosity of protein solutions. However, some of these salts have adverse effects on proteins, leading to destabilization and consequent aggregate formation (15). Thus, there is always a demand for effective formulation conditions that reduce the viscosity of a solution without compromising protein stability.

We have developed the application of ArgHCl for suppressing aggregation and adsorption of various proteins during heat treatment and oxidative refolding (16–22). These effects have been ascribed, at least in part, to the interaction of aromatic groups on arginine with the protein surface (23). We have shown that ArgHCl binds to aromatic amino acids in proteins using a crystal structure (24) and increases the solubility of low molecular weight solutes containing aromatic moieties, consistent with the above mechanism (25–29). ArgHCl does not destabilize the protein structure but decreases the probability of aggregation (30–36). Based on its effects on protein aggregation, ArgHCl might also reduce the viscosity of protein solutions by suppressing transient protein–protein interactions, thus preventing the formation of a three-dimensional network. In this work, we examined the effect of various amino acids on the viscosity of bovine and human serum albumin (BSA and HSA, respectively). HSA is a clinically important supplement for the loss of body fluid. This is the first report describing the application of amino acids for reducing the viscosity of HSA solutions.

MATERIAL AND METHODS

BSA and HSA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The purity of BSA and HSA was more than 98% and 96%, respectively. L-Alanine (Ala), L-valine (Val), L-methionine (Met), L-proline (Pro), glycine (Gly), L-serine (Ser), L-threonine (Thr), L-lysine hydrochloride (LysHCl), L-arginine hydrochloride (ArgHCl), L-histidine (His), guanidine hydrochloride (GdnHCl), sodium chloride (NaCl) were

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obtained from Wako Pure Chemical Inc. Ltd. (Osaka, Japan). All chemicals were of reagent grade and were used as received.

The sample solution was prepared as follows: Serum albumin was dissolved in deionized water, and the pH of the protein solution was adjusted to the desired values with 0.5 M NaOH or HCl. The additive solution was mixed with the serum albumin solution to achieve the appropriate concentrations of protein and additive, and the pH was then readjusted.

The viscosity of the sample solution was measured with a torsional oscillation VM-10A-L viscometer (CBC Materials Co. Ltd., Japan) at 25°C without depending on shear rate. A 1 ml sample was loaded onto the measuring microtube. The viscosity of each sample was measured at least in duplicate. Error bars in Figures depict the standard deviation of the mean of three independent experiments.

Fourier transform infrared (FTIR) spectra of the sample solutions were measured on an FT-IR 4200 spectrometer (Japan Spectroscopy Co., Ltd., Japan) using the attenuated total reflection (ATR) method. The sample solution was placed on the surface of a single-reflection ZnSe prism on an ATR accessory. Spectra were collected in the range of 1700–1500 cm^{-1} with a spectral resolution of 2.0 cm^{-1}.

RESULTS AND DISCUSSION

Examination of the solution conditions The viscosity of a protein solution is due, at least in part, to protein–protein interactions and is therefore expected to change with pH; this behavior is similar to protein solubility, which also depends on solution pH. The viscosity of a 40 mg/ml BSA solution as a function of pH was reported previously (37). The lowest viscosity was observed at around pH 4.8, near the isoelectric point (p*I*) of BSA, which is in good agreement with our experimental value (data not shown). It is clear that the viscosity is greater when BSA is positively charged at pH 4.0 and negatively charged above pH 6.0, which suggests the involvement of charge–charge repulsive interactions in network formation. Fig. 1 shows that the viscosity of the BSA solution at pH 7.4 is a function of the protein concentration up to 300 mg/ml. The solution viscosity remained nearly constant below 150 mg/ml. Further increases in protein concentration resulted in an exponential increase in viscosity to over 100 cP at 300 mg/ml.

In order to confirm the structural change by such high concentration, we measured FTIR spectra of BSA as a function of protein concentration at pH 6.8, which is the pH value of BSA solutions not adjusted, due to the avoidance of unnecessary factors (Fig. 2). The amide I and amide II regions from 1700 to 1500 cm^{-1} of the FTIR spectra were clearly observed with the BSA concentration of 75–300 mg/ml, which reflects the second structure of protein. The

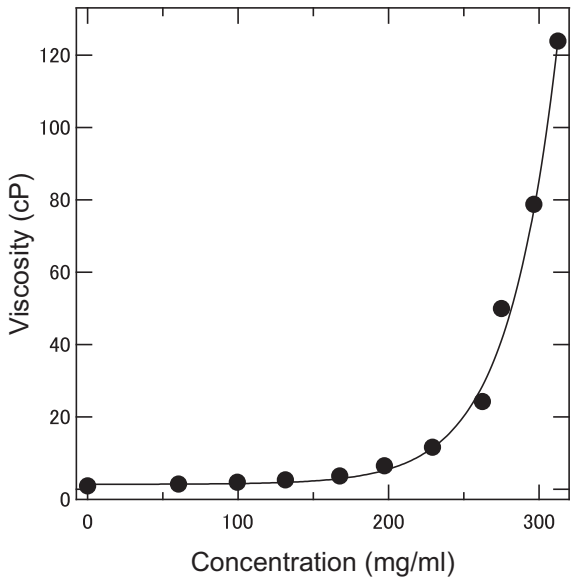

FIG. 1. Viscosity of BSA solutions as a function of the protein concentration at pH 7.4. Various concentrations of BSA were prepared at pH 7.4 and 25°C.

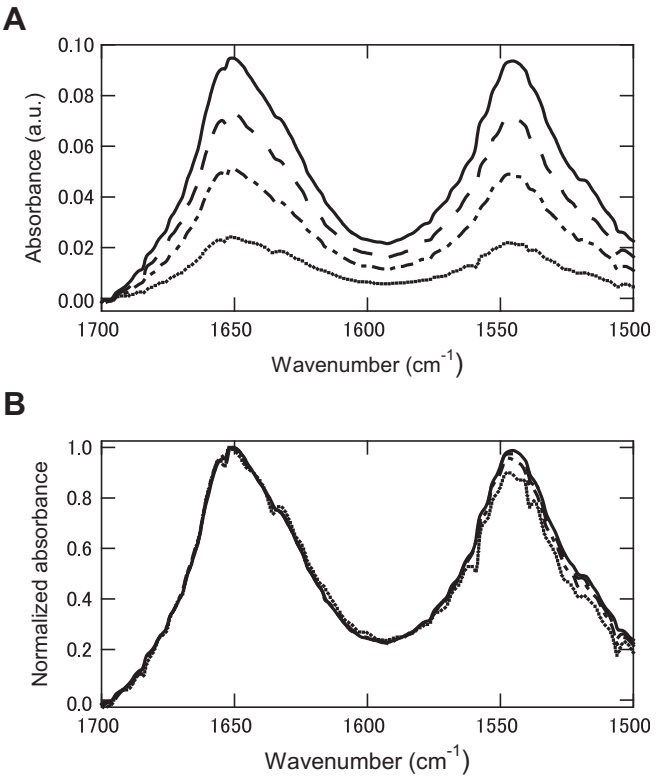

FIG. 2. FTIR spectra in the amide I and amide II regions of BSA solutions as a function of the protein concentration. Original (A) and normalized (B) spectra of BSA at pH 6.8 and 25°C: dotted line, 75 mg/ml; broken and dotted line, 150 mg/ml; broken line, 225 mg/ml; and solid line, 300 mg/ml.

maximal intensity of the amide I region at 1651 cm^{-1} increased with increasing the protein concentration. The normalized spectra of the amide I and amide II regions of BSA with the concentration of 75–300 mg/ml were identical (Fig. 2B), indicating that the secondary structure of BSA retained constant even in the presence of high protein concentration.

Effect of amino acids on the viscosity of BSA solutions Because the viscosity of BSA solution was dependent on both pH and protein concentration, we took these parameters into consideration when determining the solution conditions for evaluating the effects of various additives. We chose a pH of 7.4 because it is preferable for injectable solutions to be similar to physiological conditions. At this pH, a 275 mg/ml BSA solution showed a viscosity of ~50 cP (see Fig. 1). Any additives that lower the viscosity below this value may be acceptable for pharmaceutical applications (e.g., subcutaneous injection). Using a pH of 7.4 and a concentration of 275 mg/ml, we examined the effects of additives on the viscosity of a BSA solution.

Fig. 3 shows the viscosity of a 275 mg/ml BSA solution at pH 7.4 in the presence of 200 mM additives. The additives tested were the 10 naturally occurring amino acids shown in Fig. 3; other amino acids were insufficiently soluble in the presence of 275 mg/ml BSA. Other additives were also tested for control experiment; NaCl served as salts, and GdnHCl served as ArgHCl. Errors in the viscosity measurements may be mostly attributed to preparation errors such as alterations in protein concentration because no viscosity errors were noted when the same sample solution was measured. As shown in Fig. 3, the viscosity of the BSA solution was ~50 cP in the absence of additives. In the presence of 200 mM ArgHCl or LysHCl, the viscosity of the BSA solution decreased to 28 cP and 25 cP, respectively, corresponding to 1.9-fold and 2.0-fold respective reductions in viscosity relative to the solution without additives.