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Arginine and Lysine Reduce the High Viscosity of Serum Albumin Solutions for Pharmaceutical Injection

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Running title: Reducing viscosity with Arg and Lys

Abstract

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.

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Keywords: viscosity, arginine, lysine, serum albumin, protein-protein interaction

Introduction

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). 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 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 a FT-IR 4200 spectrometer (JASCO Corporation, 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 1,700 to 1,500 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. Figure 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 1,700 to 1,500 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 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.

Figure 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. Conversely, the other amino acids barely reduced the viscosities of the BSA solutions. The effects of NaCl and GdnHCl on viscosity were the same as those of ArgHCl and LysHCl. The observed similarity between LysHCl, ArgHCl, NaCl, and GdnHCl suggests that the ionic properties of these electrolytes may play an important role in reducing the viscosity of BSA solutions, as all of these electrolytes are monovalent ions at neutral pH.

Figure 4 shows the viscosity of 275 mg/ml BSA in the presence of 0 to 200 mM LysHCl, ArgHCl, and NaCl. The viscosity of BSA sharply decreased with increasing concentrations of ArgHCl, LysHCl, and NaCl up to ~50 mM. Above 50 mM, the viscosity of BSA appeared to reach a plateau and was nearly independent of the additives, with all additives leading to a 1.5-fold reduction in viscosity. FTIR spectra showed that the secondary structure of BSA was not altered even in the presence of 200 mM ArgHCl, LysHCl, and NaCl (Fig. 5). These data indicate that BSA does not undergo structural changes during reduction of the solution viscosity.

Effect of amino acids on the viscosity of HSA solutions

Finally, we performed the same experiments with human serum albumin (HSA) which is similar in the *pI* and the sequence to BSA, as it has commercial value as a formulation excipient and plasma expander. Figure 6 shows the viscosity of 305 mg/ml HSA at pH 7.4 in the presence of 200 mM amino acid or salt. A pattern similar to that observed for BSA was noted; the viscosity of HSA was decreased 1.6-fold by ArgHCl and LysHCl, whereas the other amino acids had marginal effects. As with BSA, NaCl and GdnHCl were as effective as, or slightly more effective than, LysHCl and ArgHCl with respect to reducing the viscosity of the HSA solution.

Molecular mechanisms of viscosity reduction by ArgHCl and LysHCl

The pH dependence of BSA solution viscosity is intriguing. The viscosity was at a minimum at approximately pH 4.8, which is near the p*I* of BSA (37). This result indicated that the protein-protein interactions in relation to solution viscosity were also at a minimum. In other words, the viscosity of BSA solutions significantly increased on both sides of the p*I*, meaning that stronger protein-protein interactions that affect solution viscosity occur when BSA acquires net charges, regardless of whether these charges are negative or positive. Although the acquisition of net charges causes overall repulsion between protein molecules, either deprotonation of carboxyl groups (below the p*I*) or protonation of His and Lys (above the p*I*) may enhance the local electrostatic interaction between BSA molecules, leading to increased formation of transient noncovalent networks and a consequent increase in solution viscosity.

We expected that protein solubility would be related to solution viscosity. Protein solubility is normally minimal at the p*I* (38). Near the p*I*, the net charge of a protein should be close to zero, causing minimal charge repulsion and enhancing protein-protein interactions. Protein-protein interactions that determine protein solubility may be related to the transient

formation of a protein network that increases solution viscosity. Thus, it was expected that the viscosity of BSA solution would be maximal near its p*I*. However, the viscosity was at a minimum near the p*I* of BSA (37). Electrostatic interactions are thought to be important in high concentration formulations of monoclonal antibodies (13, 39). Thus, this unexpected result indicated that the type of protein-protein interaction that plays a role in high viscosity is different from the type of interaction responsible for determining protein solubility.

The effects of additives were examined at the physiological pH of 7.4. At this pH, BSA has a net negative charge that apparently results in a greater viscosity than that which occurs at the isoelectric pH of ~5 (37). The current study revealed that ArgHCl or LysHCl at 200 mM significantly reduced the viscosities of both BSA and HSA solutions, although those of 50 mM may be sufficient to achieve the same level of reduction (Fig. 4). The viscosity of albumin solution was 1.6-fold lower in the presence of ArgHCl or LysHCl than in their absence. A 1.6-fold reduction in viscosity (from 50 cP to 30 cP) triggered by 200 mM ArgHCl or LysHCl at a physiological pH may be sufficient for injection. To our knowledge, this is the first report on the use of amino acids to reduce the viscosity of albumin solutions under practical conditions.

The molecular mechanisms by which ArgHCl and LysHCl reduce solution viscosity will be discussed in detail below. It is evident that LysHCl and ArgHCl as well as their salts are effective for reducing the viscosity of BSA and HSA solutions. In other words, there is no specific property of Lys or Arg that plays a key role in BSA and HSA solution viscosity. We have suggested the importance of electrostatic interactions in determining the protein-protein network, which is closely related to the high viscosity of BSA and HSA solutions. It has been reported that salts can effectively weaken such electrostatic interactions (40). Thus, both LysHCl and ArgHCl may be working simply as salts. This may be the first rare case in which ArgHCl is not more effective at suppressing protein-protein interactions compared with LysHCl or salts,

which in turn suggests less involvement of aromatic or hydrophobic interactions in the viscosity of BSA and HSA solutions. When these types of interactions are involved (e.g., protein-protein interactions that determine solubility or aggregation), ArgHCl normally exerts stronger effects than any of the other additives tested here.

In conclusion, we examined the effect of amino acids on the viscosity of highly concentrated solutions of BSA and HSA. We showed that (i) ArgHCl and LysHCl are effective at reducing the viscosity of both BSA and HSA solutions, (ii) GdnHCl and NaCl equally reduce the solution viscosity of BSA, and (iii) the ionic properties of these additives play a key role in reducing the viscosity of protein solutions. These results suggested that both ArgHCl and LysHCl maybe working simply as salts, and can effectively weaken electrostatic interaction. It should be noted that ArgHCl is one of the most used solution additive that decreases the probability of protein aggregation (30–36), adsorption on solid surface (16), and increase the solubility of water-insoluble drugs (27–29) and reduced-denatured proteins (19, 20). This is the first report that amino acids reduce the solution viscosity of HSA, which is a clinically important product that is used as a supplement for the loss of body fluid.

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Figure legends

Figure 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.

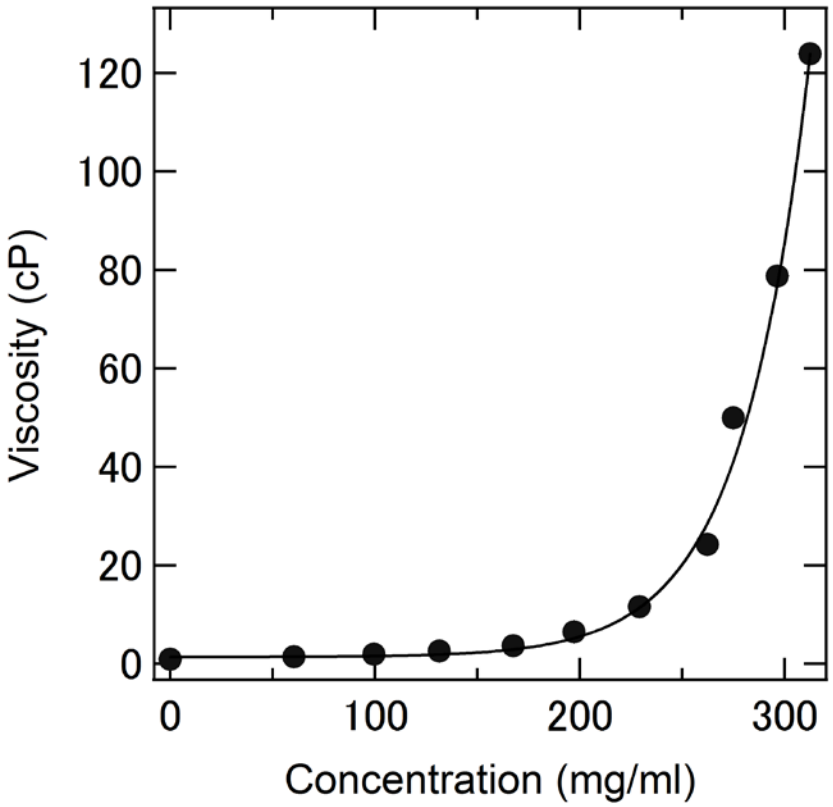
Figure 2. FTIR spectra in the amide I and amide II regions of BSA solutions as a function of the protein concentration. Original (A) and normalized (B) spectra of BSA at pH 6.8 and 25°C: 75 mg/ml (dotted line), 150 mg/ml (broken and dotted line), 225 mg/ml (broken line) and 300 mg/ml (solid line).

Figure 3. Viscosity of BSA solutions in the absence or presence of additives. Samples containing 275 mg/ml BSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars depict the standard deviation of the mean of three independent experiments.

Figure 4. Viscosity of BSA solutions as a function of additive concentration. Samples containing 275 mg/ml BSA with various concentrations of LysHCl (closed circle), ArgHCl (open square) and NaCl (closed triangle) were prepared at pH 7.4 and 25°C.

Figure 5. FTIR spectra in the amide I and amide II regions of BSA solutions with additives. Samples containing 275 mg/ml BSA with 200 mM additives were prepared at pH 7.4 and 25°C: No additives (solid line), LysHCl (broken line), ArgHCl (broken and dotted line), NaCl (dotted line).

Figure 6. Viscosity of HSA solutions in the absence or presence of additives. Samples containing 305 mg/ml HSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars depict the standard deviation of the mean of three independent experiments.



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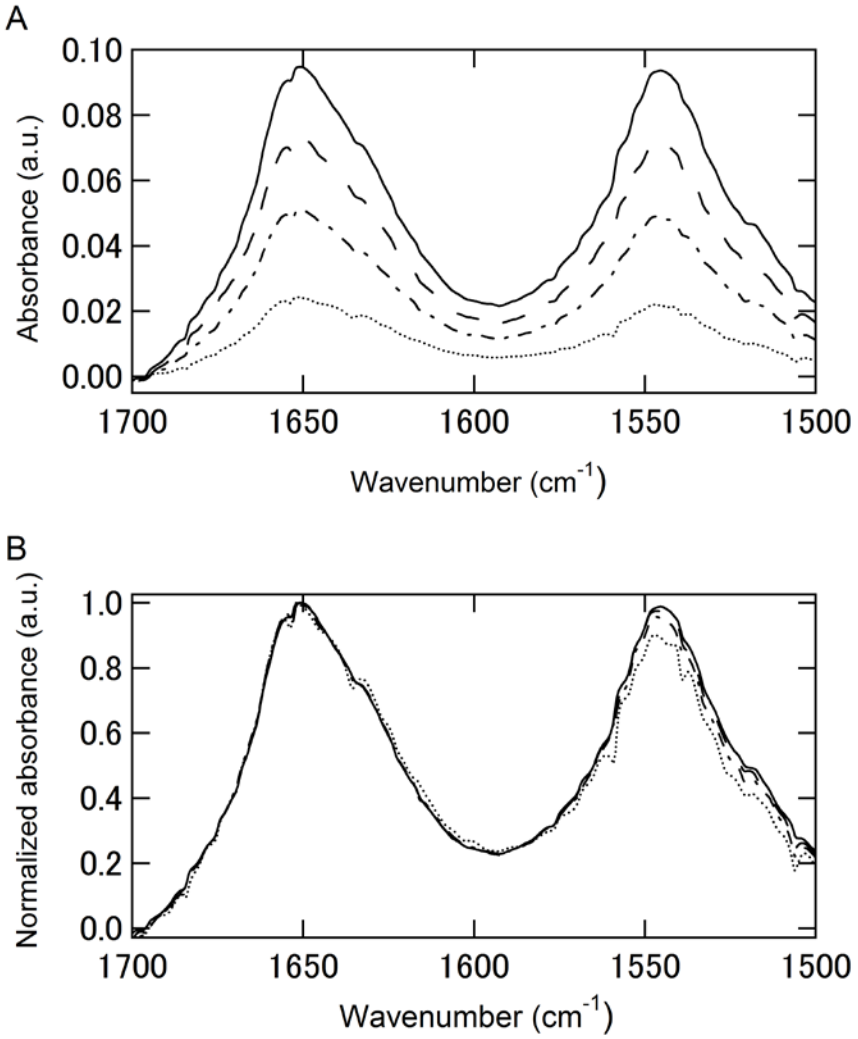

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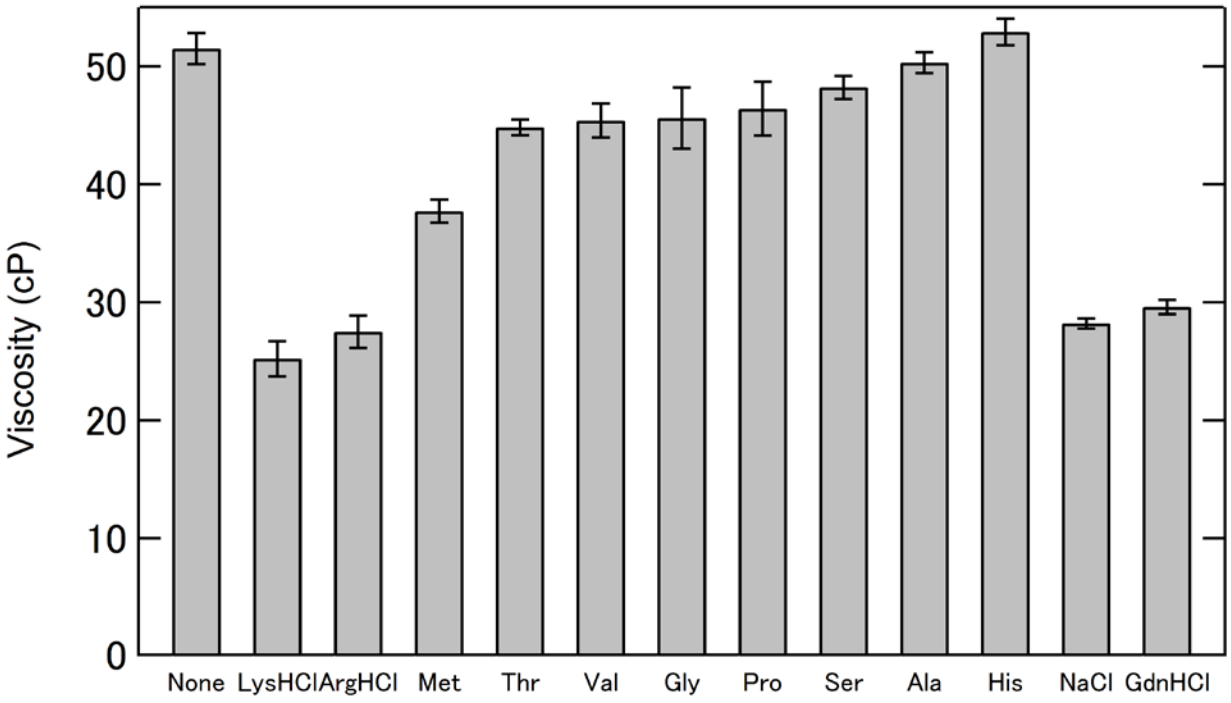

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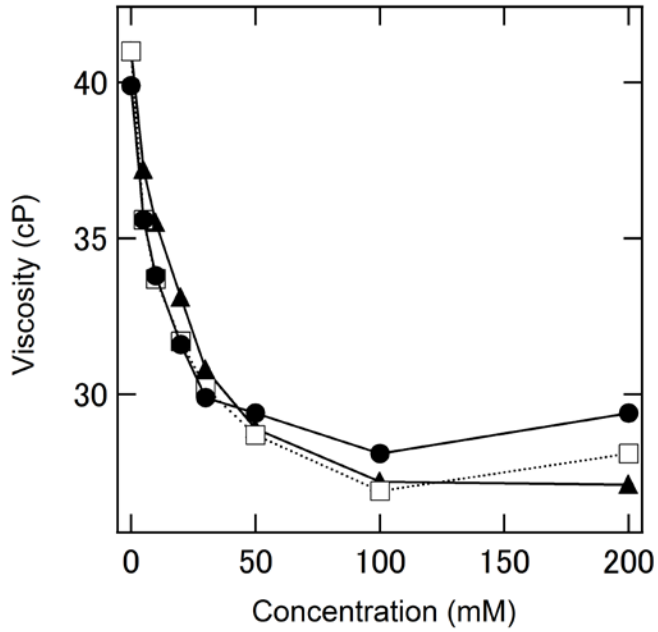

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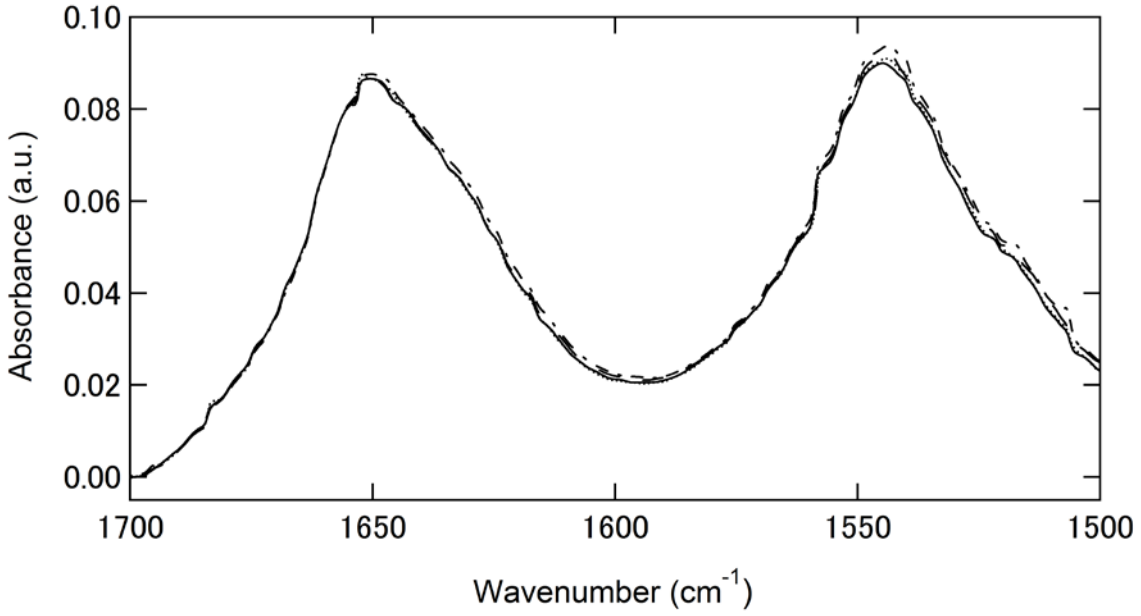

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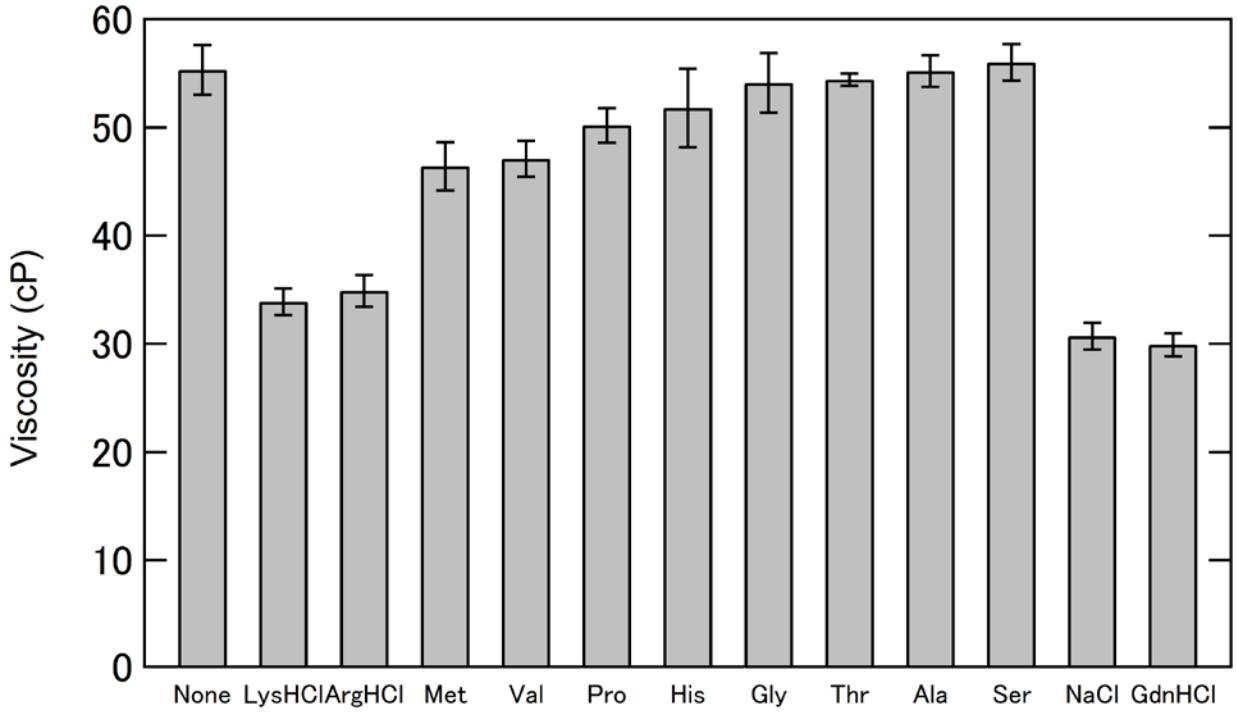

Figure 6. Viscosity of HSA solutions in the absence or presence of additives. Samples containing 305 mg/ml HSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars depict the standard deviation of the mean of three independent experiments.