

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

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18

19 **Abstract**

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

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

36

37 **Introduction**

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

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

56 We have developed the application of ArgHCl for suppressing aggregation and
57 adsorption of various proteins during heat treatment and oxidative refolding (16–22). These
58 effects have been ascribed, at least in part, to the interaction of aromatic groups on arginine with
59 the protein surface (23). We have shown that ArgHCl binds to aromatic amino acids in proteins

60 using a crystal structure (24) and increases the solubility of low molecular weight solutes
61 containing aromatic moieties, consistent with the above mechanism (25–29). ArgHCl does not
62 destabilize the protein structure but decreases the probability of aggregation (30–36). Based on
63 its effects on protein aggregation, ArgHCl might also reduce the viscosity of protein solutions by
64 suppressing transient protein-protein interactions, thus preventing the formation of a three-
65 dimensional network. In this work, we examined the effect of various amino acids on the
66 viscosity of bovine and human serum albumin (BSA and HSA, respectively). HSA is a clinically
67 important supplement for the loss of body fluid. This is the first report describing the application
68 of amino acids for reducing the viscosity of HSA solutions.

69

70

71 **Material and Methods**

72 BSA and HSA were obtained from Sigma Chemical Co. (St. Louis, MO). The purity of
73 BSA and HSA was more than 98% and 96%, respectively. L-Alanine (Ala), L-valine (Val), L-
74 methionine (Met), L-proline (Pro), glycine (Gly), L-serine (Ser), L-threonine (Thr), L-lysine
75 hydrochloride (LysHCl), L-arginine hydrochloride (ArgHCl), L-histidine (His), guanidine
76 hydrochloride (GdnHCl), sodium chloride (NaCl) were obtained from Wako Pure Chemical Inc.
77 Ltd. (Osaka, Japan). All chemicals were of reagent grade and were used as received.

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

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

87 Fourier transform infrared (FTIR) spectra of the sample solutions were measured on a
88 FT-IR 4200 spectrometer (JASCO Corporation, Japan) using the attenuated total reflection
89 (ATR) method. The sample solution was placed on the surface of a single-reflection ZnSe prism
90 on an ATR accessory. Spectra were collected in the range of 1,700 to 1,500 cm^{-1} with a spectral
91 resolution of 2.0 cm^{-1} .

92

93

94 **Results and Discussion**

95 **Examination of the solution conditions**

96 The viscosity of a protein solution is due, at least in part, to protein-protein interactions
97 and is therefore expected to change with pH; this behavior is similar to protein solubility, which
98 also depends on solution pH. The viscosity of a 40 mg/ml BSA solution as a function of pH was
99 reported previously (37). The lowest viscosity was observed at around pH 4.8, near the isoelectric
100 point (pI) of BSA, which is in good agreement with our experimental value (data not shown). It
101 is clear that the viscosity is greater when BSA is positively charged at pH 4.0 and negatively
102 charged above pH 6.0, which suggests the involvement of charge-charge repulsive interactions in
103 network formation. Figure 1 shows that the viscosity of the BSA solution at pH 7.4 is a function

104 of the protein concentration up to 300 mg/ml. The solution viscosity remained nearly constant
105 below 150 mg/ml. Further increases in protein concentration resulted in an exponential increase
106 in viscosity to over 100 cP at 300 mg/ml.

107 In order to confirm the structural change by such high concentration, we measured FTIR
108 spectra of BSA as a function of protein concentration at pH 6.8, which is the pH value of BSA
109 solutions not adjusted, due to the avoidance of unnecessary factors (Fig. 2). The amide I and
110 amide II regions from 1,700 to 1,500 cm^{-1} of the FTIR spectra were clearly observed with the
111 BSA concentration of 75-300 mg/ml, which reflects the second structure of protein. The maximal
112 intensity of the amide I region at 1651 cm^{-1} increased with increasing the protein concentration.
113 The normalized spectra of the amide I and amide II regions of BSA with the concentration of 75-
114 300 mg/ml were identical (Fig. 2B), indicating that the secondary structure of BSA retained
115 constant even in the presence of high protein concentration.

116

117 **Effect of amino acids on the viscosity of BSA solutions**

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

126 Figure 3 shows the viscosity of a 275 mg/ml BSA solution at pH 7.4 in the presence of
127 200 mM additives. The additives tested were the 10 naturally occurring amino acids shown in
128 Fig. 3; other amino acids were insufficiently soluble in the presence of 275 mg/ml BSA. Other
129 additives were also tested for control experiment; NaCl served as salts, and GdnHCl served as
130 ArgHCl. Errors in the viscosity measurements may be mostly attributed to preparation errors
131 such as alterations in protein concentration because no viscosity errors were noted when the same
132 sample solution was measured. As shown in Fig. 3, the viscosity of the BSA solution was ~50 cP
133 in the absence of additives. In the presence of 200 mM ArgHCl or LysHCl, the viscosity of the
134 BSA solution decreased to 28 cP and 25 cP, respectively, corresponding to 1.9-fold and 2.0-fold
135 respective reductions in viscosity relative to the solution without additives. Conversely, the other
136 amino acids barely reduced the viscosities of the BSA solutions. The effects of NaCl and
137 GdnHCl on viscosity were the same as those of ArgHCl and LysHCl. The observed similarity
138 between LysHCl, ArgHCl, NaCl, and GdnHCl suggests that the ionic properties of these
139 electrolytes may play an important role in reducing the viscosity of BSA solutions, as all of these
140 electrolytes are monovalent ions at neutral pH.

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

148

149 **Effect of amino acids on the viscosity of HSA solutions**

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

157

158 **Molecular mechanisms of viscosity reduction by ArgHCl and LysHCl**

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

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

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

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

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

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

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

212

213

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218

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326

327

328

329 **Figure legends**

330 **Figure 1.** Viscosity of BSA solutions as a function of the protein concentration at pH 7.4.

331 Various concentrations of BSA were prepared at pH 7.4 and 25°C.

332

333 **Figure 2.** FTIR spectra in the amide I and amide II regions of BSA solutions as a function of the

334 protein concentration. Original (A) and normalized (B) spectra of BSA at pH 6.8 and 25°C: 75

335 mg/ml (dotted line), 150 mg/ml (broken and dotted line), 225 mg/ml (broken line) and 300

336 mg/ml (solid line).

337

338 **Figure 3.** Viscosity of BSA solutions in the absence or presence of additives. Samples containing

339 275 mg/ml BSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars

340 depict the standard deviation of the mean of three independent experiments.

341

342 **Figure 4.** Viscosity of BSA solutions as a function of additive concentration. Samples containing

343 275 mg/ml BSA with various concentrations of LysHCl (closed circle), ArgHCl (open square)

344 and NaCl (closed triangle) were prepared at pH 7.4 and 25°C.

345

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

350

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

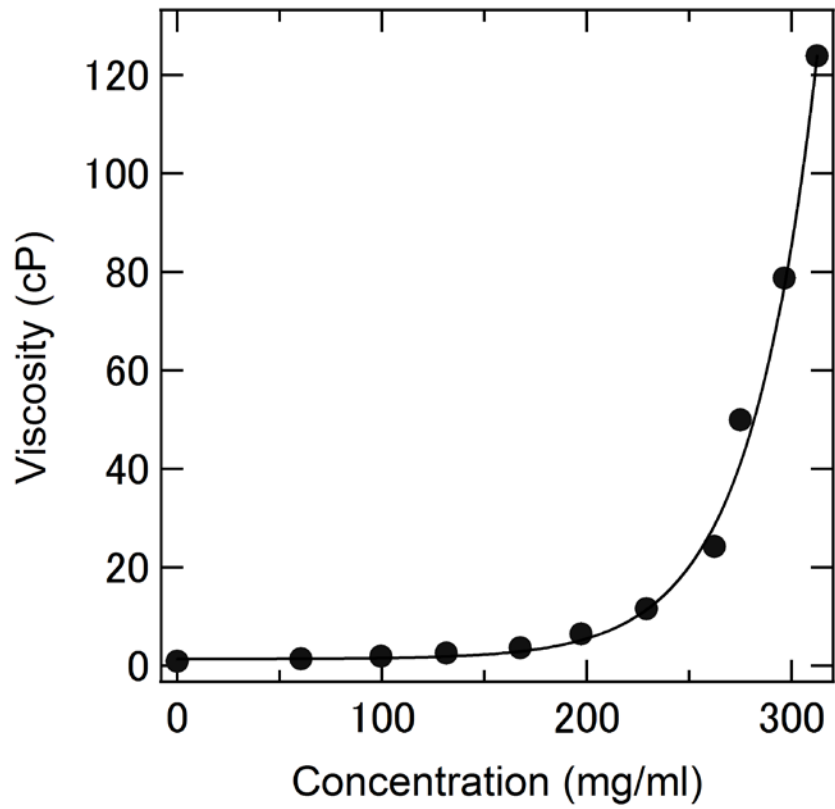


Figure 1. Viscosity of BSA solutions as a function of the protein concentration at pH 7.4. Various concentrations of BSA were prepared at pH 7.4 and 25°C.

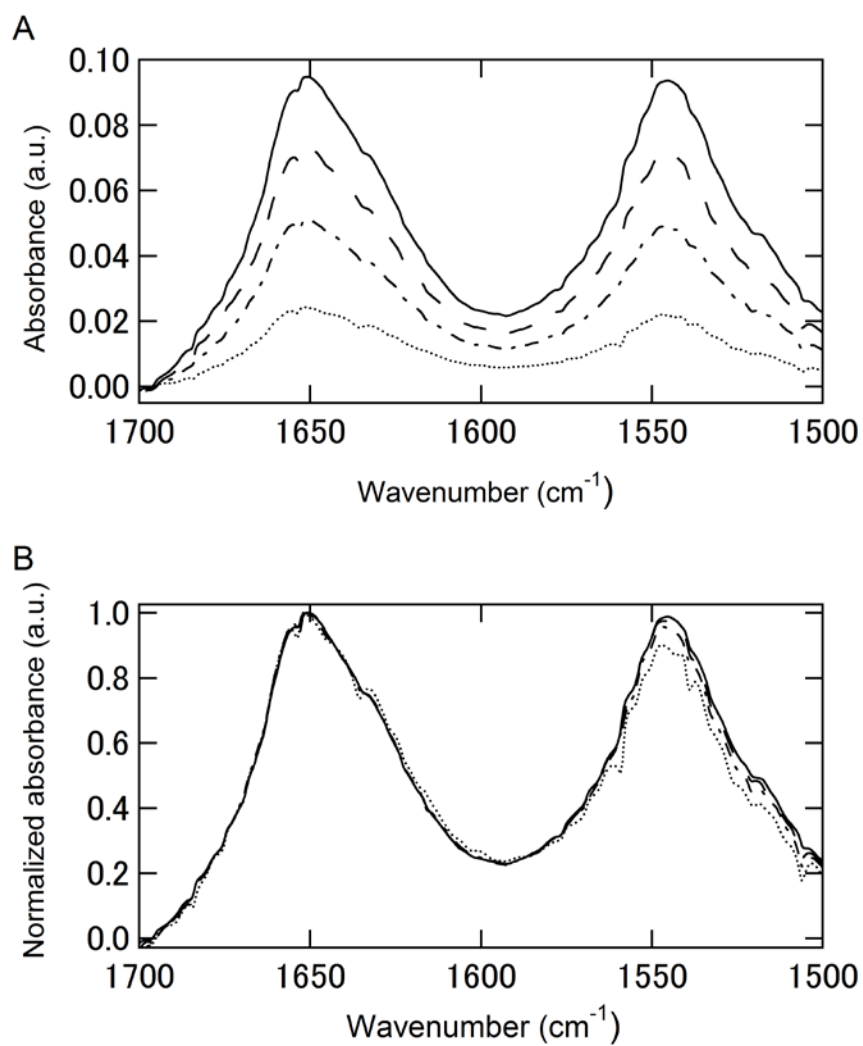


Figure 2. FTIR spectra in the amide I and amide II regions of BSA solutions as a function of the protein concentration. Original (A) and normalized (B) spectra of BSA at pH 6.8 and 25°C: 75 mg/ml (dotted line), 150 mg/ml (broken and dotted line), 225 mg/ml (broken line) and 300 mg/ml (solid line).

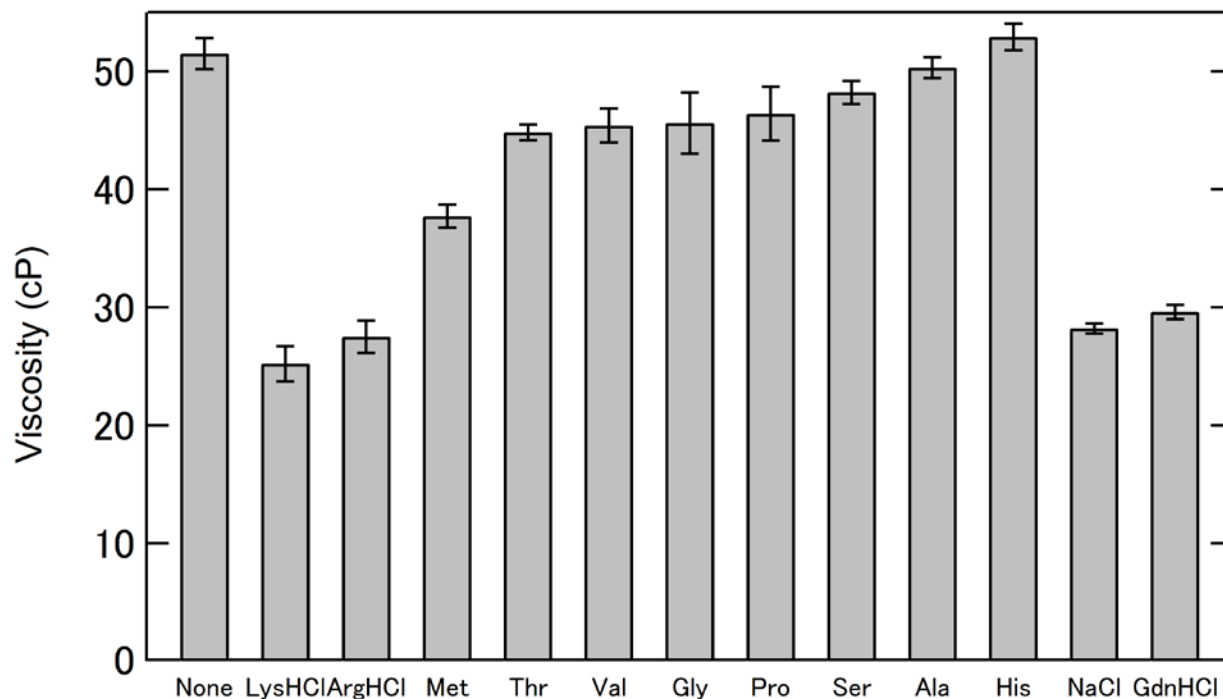


Figure 3. Viscosity of BSA solutions in the absence or presence of additives. Samples containing 275 mg/ml BSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars depict the standard deviation of the mean of three independent experiments.

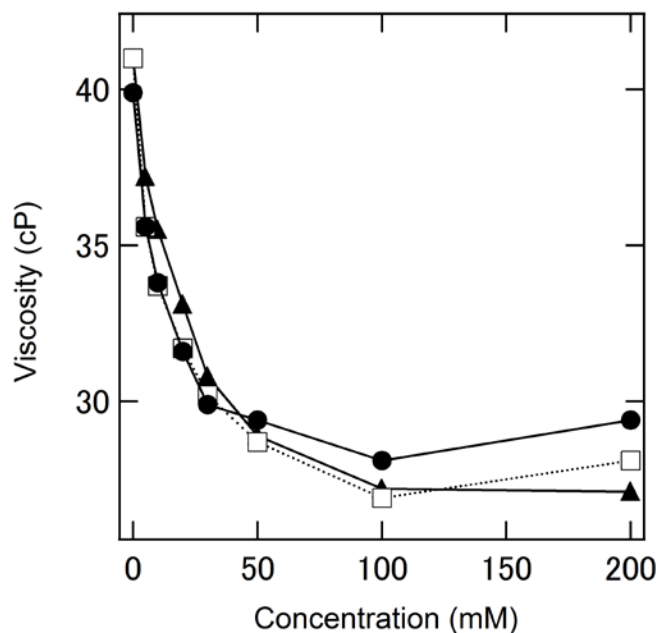


Figure 4. Viscosity of BSA solutions as a function of additive concentration. Samples containing 275 mg/ml BSA with various concentrations of LysHCl (closed circle), ArgHCl (open square) and NaCl (closed triangle) were prepared at pH 7.4 and 25°C.

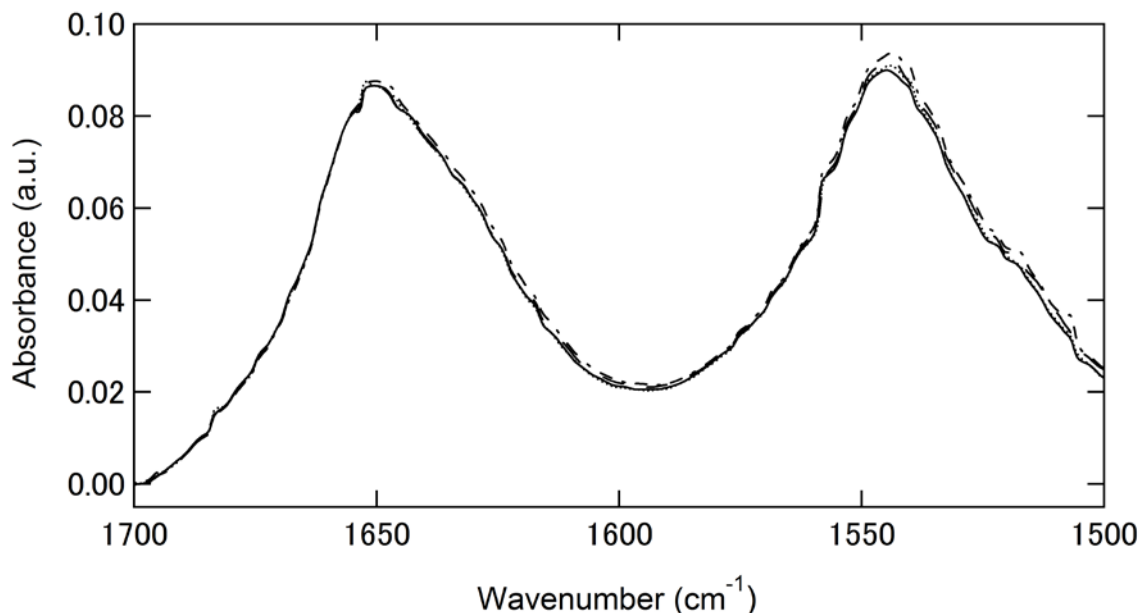


Figure 5. FTIR spectra in the amide I and amide II regions of BSA solutions with additives. Samples containing 275 mg/ml BSA with 200 mM additives were prepared at pH 7.4 and 25°C: No additives (solid line), LysHCl (broken line), ArgHCl (broken and dotted line), NaCl (dotted line).

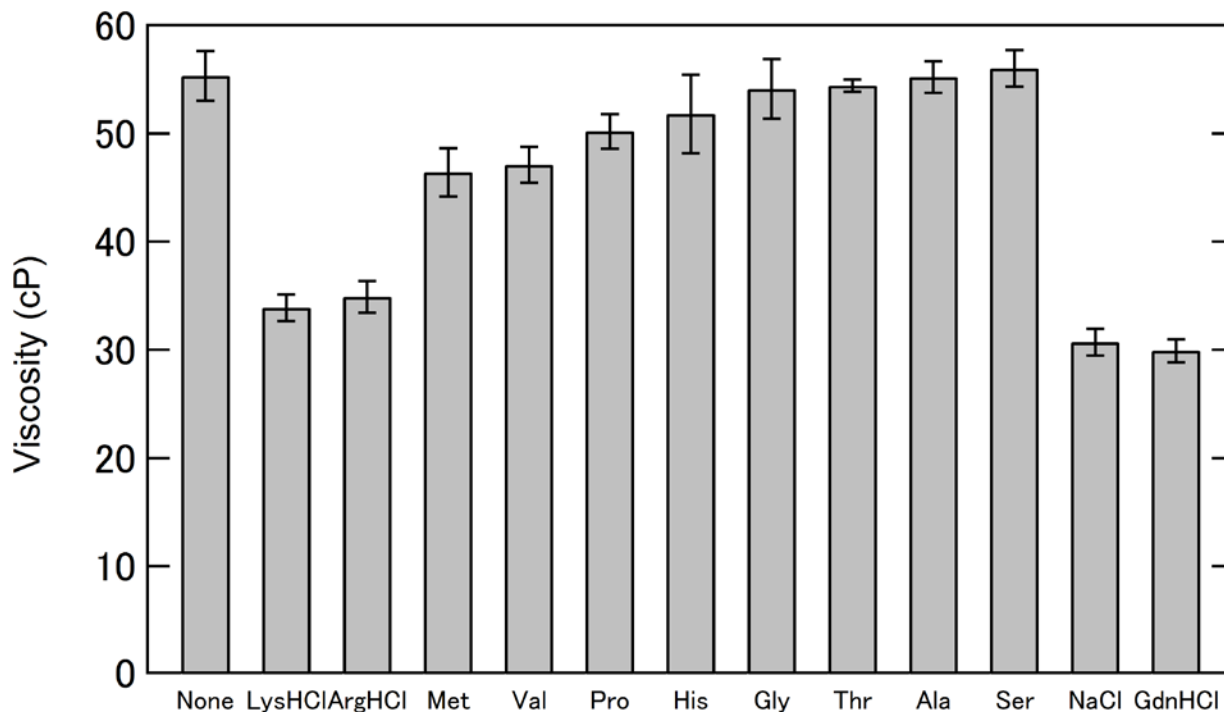


Figure 6. Viscosity of HSA solutions in the absence or presence of additives. Samples containing 305 mg/ml HSA with 200 mM amino acid or salt were prepared at pH 7.4 and 25°C. Error bars depict the standard deviation of the mean of three independent experiments.