Thermal Aggregation of Human Immunoglobulin G in Arginine Solutions: Contrasting Effects of Stabilizers and Destabilizers

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Highlights

1. Aggregation of immunoglobulin G (IgG) is a major obstacle in pharmaceutical applications.
2. Thermal aggregation pathway of IgG was mediated via soluble oligomers.
3. Arginine cannot prevent the formation of soluble oligomers but can inhibit the insoluble aggregation during heat treatment.
4. Protein stabilizer of sugars can suppress the formation of soluble oligomers and thereby increase the concentration of monomers after heat treatment.
5. The different behavior of aggregation results from the fast soluble oligomer formation and slow insoluble aggregation.

Abstract

Arginine is widely used as aggregation suppressor of proteins in biotechnology and pharmaceutics. However, why the effect of arginine depends on the types of proteins and stresses, including monoclonal antibodies, is still unclear. Here we investigated the precise processes of the thermal aggregation of human immunoglobulin G (IgG) in the presence of additives. As expected, arginine was the best additive to suppress the formation of insoluble aggregates during heat treatment, though it was unable to preserve the monomer content. A systematic analysis of the additives showed that sugars and kosmotropic ion inhibit the formation of soluble oligomers. These behaviors indicate that the thermal aggregation of IgG occurs by (i) the formation of soluble oligomers, which is triggered by the unfolding process that can be stabilized by typical osmolytes, and (ii) the formation of insoluble aggregates through weak cluster–cluster interactions, which can be suppressed by arginine. Understanding the detailed mechanism of arginine will provide useful information for the rational formulation design of antibodies.

1. Introduction

Antibody is one of the most important classes of biopharmaceutics owing to its high specificity and biocompatibility [1,2]. Antibodies are composed of multiple globular domains that assemble into a Y-shaped structure, generating multiple binding sites to themselves and other molecules [3]. Their Fab region is responsible for antigen-specific binding, and the Fc region is responsible for receptor binding [3]. Although this complex structure results in antigen specificity of the antibody with few side effects, it may be a potential source for aggregation [4–6]. Despite its high solubility, the antibody tends to form aggregates both reversibly and irreversibly due to various stresses during manufacturing, shipping, and storage processes [7]. The aggregation of proteins is known to cause unexpected immunogenic reactions [8]. Therefore, understanding the mechanism of antibody aggregation and the method to prevent aggregation is critical for developing safer products.

Protein aggregation can occur in two different manners, i.e., via aggregation of native or nonnative structures [9,10]. The first type of aggregation is mediated through attractive intermolecular interactions between molecules of native structure, in particular at higher protein concentrations (the so-called colloidal aggregation) [11]. This mode of aggregation is often observed among antibodies and is a cause of their anomalous viscosity [12]. The aggregation of antibodies with native structure can be suppressed by chaotropes that reduce the attractive interactions [13]. The second type of aggregation is mediated by partial or extensive changes in the conformation caused by various stresses, e.g., elevated temperature, addition of destabilizing solvent additives, and mechanical stresses [14]. This type of aggregation is usually irreversible and can be suppressed by either stabilizing the native structure against conformational changes [15] or suppressing the intermolecular interaction between the (partially) unfolded proteins [16]. The second type of aggregation can occur, for example, among antibodies during manufacturing processes, e.g., elution from protein A by a low pH buffer [17].

Solvent additives play an important role in suppressing protein aggregation [18,19]. Various types of solvent additives that either enhance protein stability or suppress protein aggregation have

been investigated on globular proteins [18]. The protein stabilizers such as sodium sulfate [20] and sugar [21] interact favorably with water molecules, whereas the protein denaturant destabilizers such as guanidine [22] and urea [23] interact favorably with hydrophobic surfaces of the proteins, reducing the protein–protein interactions. Arginine has the unique property of suppressing protein aggregation without causing denaturation [24–26]. Although the knowledge of a cosolvent has been accumulated [27], it cannot be readily applied for antibodies due to their structural complexity. In this study, we investigated the effects of known protein stabilizers or destabilizers as well as arginine on the heat-induced aggregation of human immunoglobulin (IgG).

2. Material and methods

2.1. Material

Human IgG was obtained from MBL life science (Nagoya, Japan). Sodium lauroyl glutamate was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). Arginine hydrochloride, sodium chloride, sodium phosphate, sodium thiocyanate (NaSCN), sodium sulfate, guanidine hydrochloride (Gdn), urea, glucose, trehalose dihydrate, xylitol, ethanol, and ethylene glycol were from Wako Pure Chemical Inc., Ltd. (Osaka, Japan). All chemicals used were of reagent grade and used as received. IgG was dissolved in and dialyzed against pure water to remove salts before the following experiments. The protein concentration of the above stock IgG solution was spectrophotometrically determined using a UV-vis spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, USA).

2.2. Determination of IgG monomer and oligomer concentrations

The stock IgG solution in water was diluted to 2.0 mg/ml sample solution containing solvent additives at the indicated concentration and 100 mM Na-phosphate buffer (pH 7.0). The diluted samples were heated at 75°C for 2 min or longer and cooled at 4°C for 5 min. The samples were then centrifuged at 18,800 *g* for 30 min to remove the insoluble aggregates. After centrifugation, the

supernatant was subjected to size exclusion chromatography on a high-performance liquid chromatography (HPLC) using a size exclusion column (Yarra SEC 3000; Phenomenex, Torrance, CA). The column was equilibrated with a running buffer containing 100 mM Na-phosphate buffer (pH 7.0) and 200 mM arginine at a flow rate 1.0 ml/min. A 50 μl aliquot of the supernatants was loaded into the column. The elution was monitored at 280 nm, and the concentration of IgG monomer was calculated from the peak area.

The amount of soluble IgG oligomers was determined as described below. A 10 μl aliquot of the supernatants was diluted with 10 μl of 100 mM Na-phosphate buffer (pH 7.0) containing 8 M Gdn to eliminate the contribution of light scattering of the soluble oligomers to the total absorbance. The absorbance of the mixture was determined using a UV-vis spectrophotometer. This absorbance corresponds to the total protein amount, i.e., the amounts of the monomers and the soluble oligomers. The amount of soluble oligomers was determined by subtracting the monomer amount determined from the size exclusion analysis. The amount of insoluble aggregates was calculated from the total protein subjected to the heat treatment.

2.3. Thermal denaturation curve

The thermal denaturation curve of IgG was plotted by near-UV-CD measurement using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 1-cm path-length quartz cell. Briefly, 0.2 mg/ml IgG solution containing the 100 mM Na-phosphate buffer in the presence or absence of 1 M additives was loaded onto the cell. The 2-ml samples in the cell were heated at a rate of 1°C/min increment and monitored at 292 nm.

3. Results

IgG at 2.0 mg/ml was heated at 75°C for 2 min in the absence and presence of 500 mM arginine and subjected to size exclusion chromatography. Figure 1 shows the chromatogram of the control IgG, i.e., without heating (solid line). The size distribution was identical in the absence (A)

and presence (B) of arginine, indicating no effects of arginine on the control sample. Nearly 100% of the loaded IgG was eluted as a monomer at 8–9 min. When heated in the absence of arginine, the monomer peak was greatly reduced (A) and a new peak appeared at 5–6 min corresponding to the soluble IgG oligomers. Figure 1A clearly shows that the total peak area was far less than that of the control, consistent with the observed precipitates, and hence, that the heating at 75°C in the absence of arginine resulted in extensive aggregation with a consequent precipitation. In the presence of 500 mM arginine (B), the monomer peak was similarly reduced by the heat treatment, indicating that it does cause protein aggregation. However, the peak area at 5–8 min corresponding to the soluble oligomers was much greater that that observed in the absence of arginine. This is consistent with the few precipitates when heated with arginine than without arginine. Thus, it is evident that while arginine is effective in dispersing heat-generated aggregates into smaller soluble oligomers, it is not effective in preventing the association of monomeric IgG.

The effects of various additives on the thermal aggregation of IgG were examined in the presence of 500 mM arginine, i.e., the samples were heated at 75°C for 2 min. Reduction of the monomer content was examined in the absence and presence of 500 mM arginine as a function of incubation time. Figure 2 plots the change in the monomer content in the absence (solid circles) and presence (open squares) of arginine. The plots completely overlapped with each other, indicating that the addition of 500 mM arginine showed no impact on the formation of IgG aggregation. The monomer content was reduced to ~25% after heating for 2 min and to ~0% after heating for 20 min regardless of the absence or presence of arginine.

The mass balance of IgG after heat treatment is shown in Figure 3A. While the content of the soluble oligomers (gray bar) was similar to the monomer content (black bar, ~0.5 mg/ml) in the absence of arginine, the content of the soluble oligomers increased 3-fold to ~1.5 mg/ml. As a consequence, there were few precipitates in the presence of arginine (white bar) compared to those with ~0.9 mg/ml insoluble aggregates (left panel of Figure 3A). One possible explanation is that heat treatment leads to a different protein conformation in the presence of arginine from that in its absence,

which still causes a similar IgG association but reduces further aggregation of the soluble oligomers.

The distribution of monomers, soluble oligomers, and precipitates was similarly determined by size exclusion chromatography and UV-vis spectrophotometer. Figure 3B shows the effects of various salts with 500 mM arginine. It was observed that the addition of these salts to 500 mM arginine solution increased, though slightly, the insoluble aggregates (white bar) relative to the amount with arginine alone, suggesting that the reduction of electrostatic repulsion between IgG may be responsible for the formation of insoluble aggregates during heat treatment. Sodium chloride showed no change in the monomer content (black bar), decreased slightly the soluble aggregates (gray), and increased the precipitates (white). This may be due to a weak salting-out effect of this salt, leading to small-enhanced aggregation. NaSCN, known as a salting-in salt, further decreased the monomer content and increased both soluble and insoluble aggregate contents. Thus, this salt appeared to enhance the aggregation of monomeric IgG and thereby further reduce the monomer content. This may be due to the destabilizing effects of NaSCN. Interestingly, sodium sulfate (Na_2SO_4), a known salting-out salt, increased the monomer content, which is in contrast to the effects of NaSCN. It is likely that this salt stabilized the IgG structure against heat denaturation and thereby prevented IgG from monomeric association of the heat-denatured state. However, it acted as a salting-out salt on soluble oligomers, as it further decreased the soluble oligomers and increased the insoluble aggregates.

Figure 3C shows the results of the effects of various additives at the indicated concentration in the presence of 500 mM arginine after 2-min heat treatment. Gdn even at 200 mM was sufficient to further reduce the monomer content, similar to the results with NaSCN. Moreover, similar to NaSCN, 200 mM Gdn enhanced the formation of both soluble oligomers and insoluble aggregates. Urea at 1 M showed a similar trend to that of 200 mM Gdn and 500 mM NaSCN. Sugars (glucose and trehalose) and polyhydric alcohol (xylitol) at 200 mM showed a similar trend with each other, increasing the monomer content and decreasing the soluble oligomer content, which were similar to the effects of NaCl and Na_2SO_4. Thus, their effects may be explained by stabilization of the protein

structure against heat denaturation [18,28]. Ethanol at 10% resulted in no remaining monomers with the formation of both soluble oligomers and insoluble aggregates. This is most likely due to enhanced denaturation of IgG by ethanol even at 10%. Additional polyhydric alcohols (glycerol and ethylene glycol) at 10% resulted in opposite effects. Glycerol increased the monomer content due to its stabilizing effects [29], whereas ethylene glycol reduced the monomer content due to its destabilizing effects. A similar result was reported using lysozyme [30]. Both are polyhydric alcohols, but they behaved differently, i.e., ethylene glycol as a destabilizing additive and glycerol as a stabilizing additive. Thus, it does appear that the effects of the polyhydric alcohols on the stability are correlated with the number of OH group [31].

It has been shown that sodium lauroyl glutamate is a mild detergent and can solubilize insoluble proteins [32]. Figure 4 shows the monomer and soluble oligomer contents of IgG as a function of lauryl glutamate concentration when heated at 75°C for 2 min in the presence of 500 mM arginine. As the concentration of lauryl glutamate was increased, the monomer content gradually decreased (white circles) concomitant with increasing soluble oligomer content (solid circles). There were few monomers left with 0.4% lauroyl glutamate due to its denaturing effects.

The results described above indicated that the stabilizing additives increased the monomer content in the presence of 500 mM arginine. Their effects were then examined in the absence of arginine and compared with the results in the presence of 500 mM arginine. Figure 5A shows the effects of 0–1000 mM arginine on the monomer content after heating at 75°C for 2 min (closed circles). The monomer content was independent of the arginine concentration and reduced to ~30% of the total protein. Glycerol, which increased the monomer content when heated in the presence of 500 mM arginine, was ineffective by itself regardless of the concentration (closed triangles). On the contrary, trehalose was highly effective in increasing the monomer content, which nearly linearly increased with the concentration (open squares), consistent with its effects in the presence of 500 mM arginine. This is most likely due to its effectiveness as a protein stabilizer. In fact, glucose (open circles), xylitol (closed squares), and sodium sulfate (open triangles) showed a more or less similar

trend. All of them increased the monomer content, the magnitude of which was between those of trehalose and arginine/glycerol. Figure 5B shows the results of the soluble oligomer content. Arginine was most effective in increasing the soluble aggregates (closed circles). Sodium sulfate showed little effects on the soluble oligomer content up to 500 mM (open triangles). However, this salt at 750 mM, despite increasing the monomer content, resulted in reduction of soluble aggregates. In fact, 1000 mM sodium sulfate resulted in spontaneous precipitation at room temperature even before heating; no data were obtained at 1000 mM sodium sulfate as shown in Figure 5. Glycerol, which showed no effects on the monomer content, resulted in a linear increase in the soluble oligomer content (close triangles). Trehalose (open squares), which showed a linear increase in the monomer content, showed a bell-shaped concentration dependence. It increased the soluble oligomers at 250 mM followed by a gradual reduction. Glucose (open circles) and xylitol (close squares) showed a more or less similar trend, slightly increasing the soluble oligomers.

Next, thermal denaturation experiments were carried out to assess whether some of the structure-stabilizing additives at 1000 mM in fact stabilized the IgG used in this study. Figure 6 shows the change in ellipticity monitored at 292 nm with increasing temperature. For the control (without additive), the CD intensity sharply decreased at above ~75°C, indicating that the temperature used to induce aggregation was at the edge of thermal melting of the IgG. This temperature increased in the order of glycerol < xylitol < trehalose and decreased to ~70°C with arginine. It is noticeable that the CD intensity at 292 nm did not decrease to zero and instead became extremely negative, suggesting a possibility that the thermal denaturation induced a new tertiary structure with a negative CD intensity at 292 nm. However, when monitored at 350 nm, the CD intensity still went to negative. Since there is little absorbance at 350 nm for IgG or any proteins, this negative CD is not due to the formation of new structures but must be due to light scattering.

4. Discussion

In this study, we have investigated the effects of various additives on the thermal aggregation of IgG. Mechanistic understanding of the observed thermal aggregation and the effects of

each solvent additive may be explained using a scheme depicted in Figure 7, in which the aggregation is shown to be mediated by conformational changes at 75°C. It is unlikely that the observed results involve reversible colloidal aggregation in such a low IgG concentration of 2.0 mg/ml. In addition, the IgG samples were heated at 75°C, which facilitates the unfolding of IgG. Based on the reported weaker stability of Fab domain [4], it is likely that this domain first unfolds (i), which triggers the formation of soluble oligomers (ii). Further heating causes additional unfolding (iii), likely on a more stable Fc domain, leading to the last step (iv), i.e., aggregation via the Fc domain. The formation of soluble oligomers was shown to occur by other stresses such as acid exposure [33], agitation [34,35], and lyophilization [36]. Thus, attention should be paid on the formation of soluble oligomers in the process of immunoglobulin aggregation.

The additives used in this study showed differential effects on the immunoglobulin aggregation (Figure 2). The most intriguing observation is that arginine showed no effects on the amount of monomers. Namely, arginine was unable to preserve the monomer content against heat treatment. However, it did suppress the formation of insoluble aggregates (Figure 3A). Based on the thermal unfolding data (Figure 6B), arginine may facilitate partial unfolding (i), as it lowers, though slightly (a less degree), the melting temperature. Nevertheless, arginine showed no impact on the monomer content, indicating the competition between facilitation of unfolding and the suppression effects of aggregation. The observed stabilization of soluble oligomers (Figure 3A) should be imposed on the next steps (iii) and (iv). Considering the slight destabilizing effects of arginine, it is highly unlikely that arginine inhibits step (iii). Therefore, arginine should inhibit the last step (iv). It is interesting that while arginine cannot inhibit the intermolecular interaction between Fab domains (ii), it can inhibit the other intermolecular interaction between Fc domains (iv). Arginine has been suggested to suppress weak molecular interactions, which in turn suggests that the molecular interaction in (ii) may be stronger than that in (iv). A similar trend was observed with a monoclonal antibody, which was subjected to mild heat stress at low pH in the absence and presence of arginine at high concentration (personal communication). Arginine was ineffective in preserving the

monomeric antibody but was effective in preserving the soluble oligomers against the above treatment.

How about other additives? The thermal denaturation curve results showed that the denaturation can be suppressed by xylitol, trehalose, and glycerol. The observed increase in the monomer content by trehalose and xylitol as well as glucose and sodium sulfate may be explained by the stabilization of the native monomer. However, no stabilization of the monomers by glycerol alone suggests the involvement of other mechanisms. Their effects, except for sodium sulfate, on the soluble oligomers are complex and may be due to a balance between their structure stabilization effects and enhancement of protein–protein interactions. The effect of sodium sulfate is due to its overwhelming salting-out effects on the unfolded IgG, leading to the formation of insoluble aggregates or precipitates.

In summary, this study demonstrated the pathway of heat-induced aggregation of IgG and the effects of aggregation suppressors or protein stabilizers. Unlike the globular protein, the thermal aggregation pathway of IgG shows multiple steps, including the soluble oligomer state. The selection of additives strongly influences each process depending on its mechanisms. This study will be useful for the rational formulation design of immunoglobulins.

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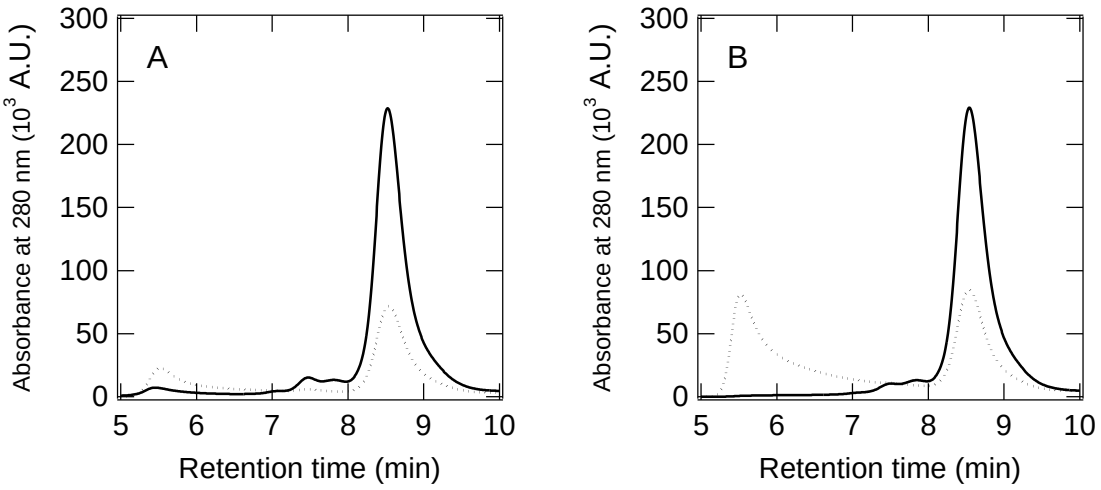

Fig. 1. Size exclusion chromatography of 2.0 mg/ml IgG solution containing 100 mM Na-phosphate buffer (pH 7.0) in the (A) absence or (B) presence of 500 mM arginine. The sample was heated at 75°C for 0 min (solid line) and 2 min (dotted line).

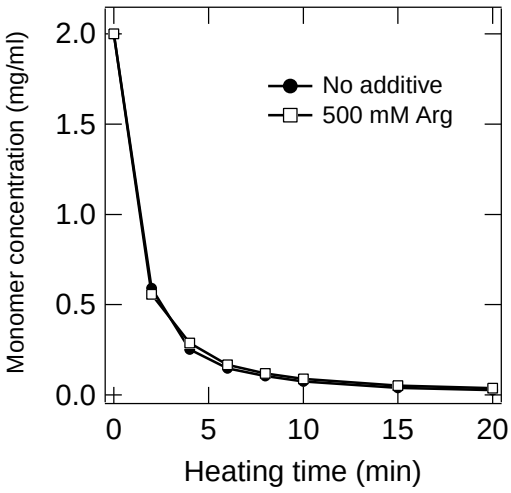

Fig. 2. Monomer concentration after the heat treatment. IgG solution of 2.0 mg/ml containing 100 mM Na-phosphate buffer (pH 7.0) in the absence (closed circles) or presence (open squares) of 500 mM arginine was heated at 75°C for 0–20 min. The monomer concentration was calculated from the peak of size exclusion chromatography.

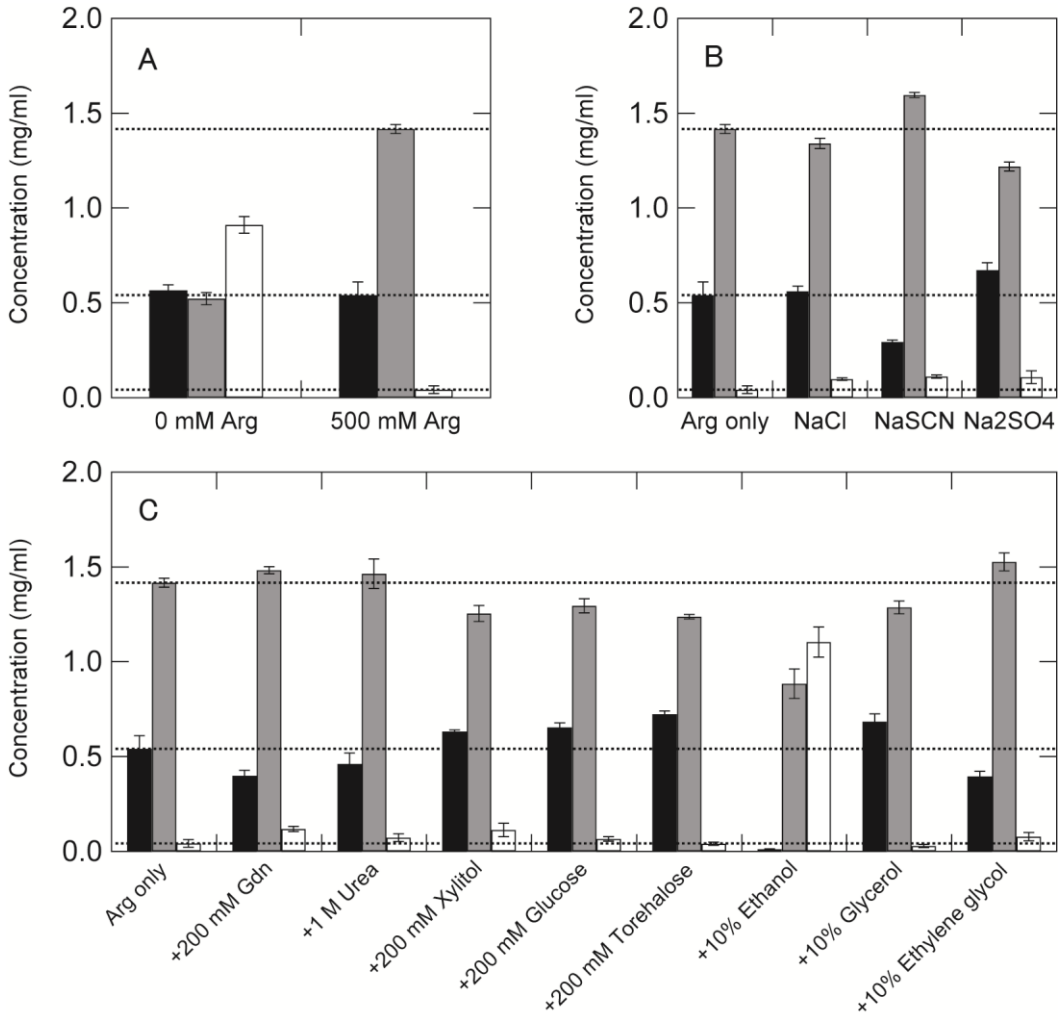

Fig. 3. Concentration of IgG after the heat treatment. Each solution containing 2.0 mg/ml IgG with various additives (pH 7.0) was heated at 75°C for 2 min, and then the concentrations of the monomer (black), soluble oligomer (gray), and insoluble aggregate (white) of IgG at 2 min were plotted. (A) Comparison of 0 and 500 mM arginine as an additive. (B) Comparison of 500 mM inorganic salts with 500 mM arginine. (C) Comparison of denaturants, osmolytes, and organic compounds with 500 mM arginine. The dotted lines indicate the result in the presence of 500 mM arginine for visual comparison. The experiment was replicated three times, and the error bars indicate the standard deviation.

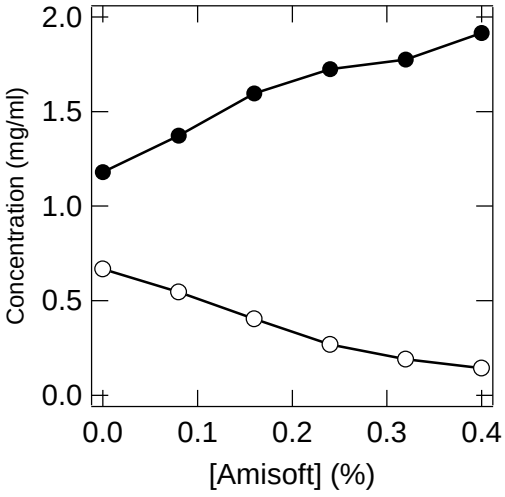

Fig. 4. Concentration of monomer (open circles) and soluble aggregate (closed circles) of IgG solution containing 0%–0.4% sodium lauroyl glutamate with 500 mM arginine after the heat treatment at 75°C for 2 min.

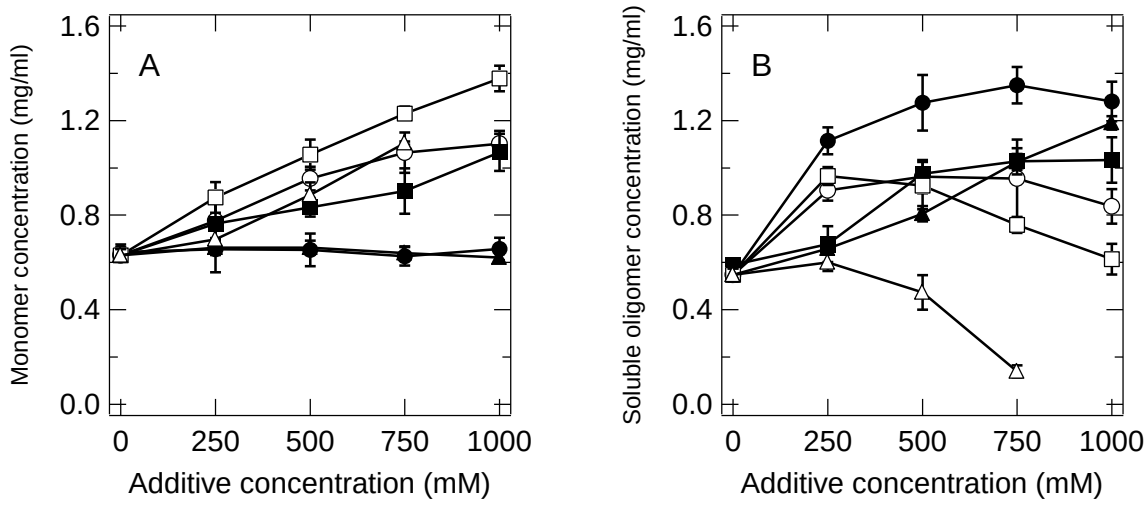

Fig. 5. Concentration of monomer (A) and soluble oligomer (B) of IgG after the heat treatment at 75°C for 2 min. The solutions contained 2.0 mg/ml IgG, arginine (closed circles), glucose (open circles), xylitol (closed squares), trehalose (open squares), glycerol (closed triangles), and sodium sulfate (open triangles). The measurements were performed three times, and the error bars depict the standard deviation of the mean.

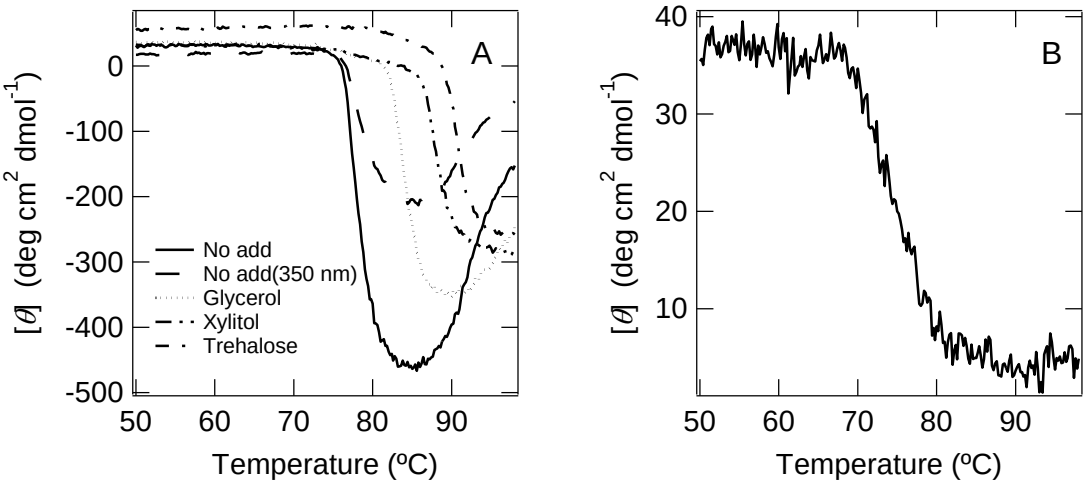

Fig. 6. Thermal denaturation curve of 0.2 mg/ml IgG in the presence or absence of additives. (A) No additive (solid line), no additive measured at 350 nm (dashed line), 1000 mM glycerol (dotted line), 1000 mM trehalose (single dotted chain line), 1000 mM xylitol (double dotted chain line). (B) 1000 mM Arg. The circular dichroism was measured at 292 nm.

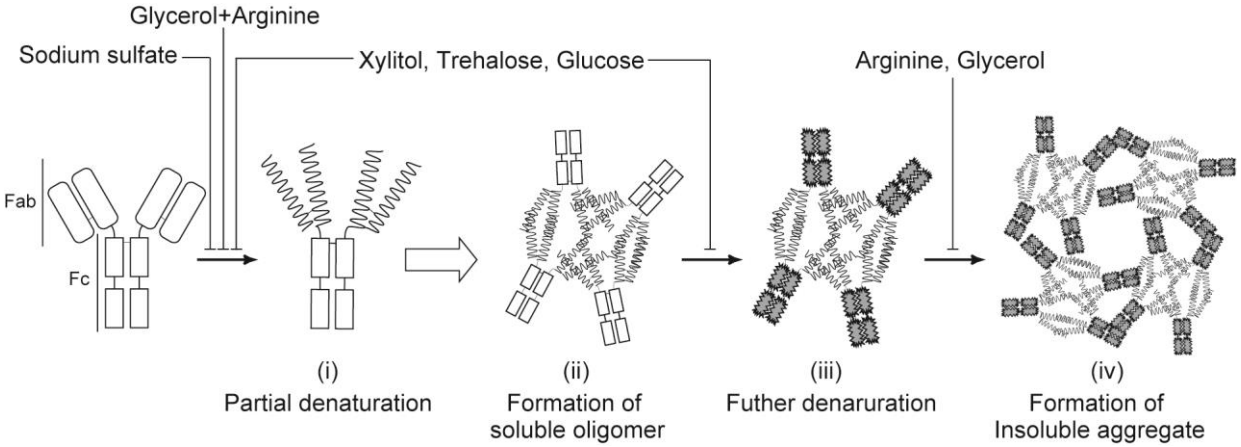

Fig. 7. Schematic illustration of the aggregation of IgG and the effect of additives.