Turbidity and Hardness of a Heat-induced Gel of Hen Egg Ovalbumin

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The turbidity and hardness of a heat-induced gel prepared from ovalbumin were examined at various pHs and ionic strengths. Depending on the conditions of the medium, a transparent solution, transparent gel, turbid gel, or turbid suspension was obtained by heating. The hardness was a maximum with the conditions that gave a transparent or slightly turbid gel. The gel and coagulums were solubilized by 1% SDS, but not by 6 M urea or 50 mM mercaptoethanol. The solution obtained by SDS treatment contained polymers shorter than octamers.

The heat-induced gel of egg white is usually turbid and firm. Ovalbumin, the major protein in egg white, gives the same kind of gel on heating. Turbidity and firmness are the results of denaturation, aggregation and coagulation of the protein molecules, and these are dependent on the environmental conditions.1 ~4) Heating the ovalbumin molecule forms a coagulum, accompanied by the formation of a rigid gel structure. Therefore, turbidity is often used as an index for the gelation of ovalbumin. However, gelation is not necessarily accompanied by turbidity of the gel. Both a turbid and transparent gel can be made from bovine serum albumin5) and other proteins6) with different gelling conditions.

Egelandsdal7) investigated the pH dependence of the rigidity of a heat-induced gel of ovalbumin and concluded that ovalbumin gelling was governed by electrostatic force. Hegg et al.8) reported that the manipulation of changes in pH, the type of salt, salt concentration, or the addition of detergents affected the thermal aggregation of proteins. These workers also suggested that ovalbumin could remain transparent after heating under certain conditions, and that the intermolecular interaction of ovalbumin was affected by small changes in the molecular environment, such as in pH or ionic strength. Consequently, regulation of the heating conditions might affect the turbidity and firmness of this gel. In this study, we controlled the state of the pH and ionic strength of the solvent medium, in particular, to find the best conditions for preparing a transparent, firm gel from an ovalbumin solution by heating. If such a gel could be readily prepared from egg white, new applications of egg white in the food industry could be developed. We also studied the interaction of ovalbumin molecules during the formation of the gel by heating to better understand the mechanism for gelation.

MATERIALS AND METHODS

Ovalbumin and other reagents. The ovalbumin was purified from fresh egg white by the ammonium sulfate procedure of Soerensen.9) The ovalbumin was recrystallized at least three times, and dialyzed against distilled water containing 0.03% NaN₃ and 0.1 mM Na₂ EDTA. The distilled water used had been bubbled with N₂ gas to remove and dissolved O₂. The purified ovalbumin preparation showed a single band on SDS polyacrylamide gel electrophoresis, and one main negative peak was found by

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differential scanning calorimetry. From these findings, we estimated the contamination by s-ovalbumin and its intermediates at less than 0.5% (w/v).

**Measurement of the protein concentration and adjustment of pH.** Ovalbumin concentrations were calculated from the absorbance at 280 nm, based on the value of E\textsubscript{1cm}\textsuperscript{\textdegree} = 7.12.\textsuperscript{10} The pH of the sample was adjusted by the addition of small amounts of a 1 N NaOH or HCl solution using a pH meter.

**Measurement of conductivity.** The electrical conductivity of the ovalbumin solution was measured with a conductivity meter (CD-35M2, M & S Instruments Inc.) at 25°C.

**Measurement of turbidity.** To 2.0 ml of a 5% (w/v) ovalbumin solution in a special test tube (2.5 i.d. x 6 mm) for turbidity measurement was added 0.5 ml of a concentrated NaCl solution or distilled water. This sample was heated for 1 hr at 80°C, and then cooled by tap water (25°C) for 3 hr. The test tube containing the sample was put directly in the cell holder of a Shimadzu (Kyoto) UV-240 spectrophotometer. The absorbance of the sample measured at 600 nm was the value for turbidity.

**Measurement of hardness.** A 3.0 ml sample of a 5% (w/v) ovalbumin solution in a vessel (25 i.d. x 6 mm) made of stainless steel was sealed with rubber packing, sheet glass, and clips. The sample was heated in a water-bath (80°C) for 1 hr. After cooling with tap water for 3 hr, the hardness of the sample was measured with a Rheoner RE-3305 (Yamaden Co., Ltd., Tokyo). A plunger with a diameter of 8 mm was attached, and the sample was placed on the plate, which was set to move at 1 mm/sec in the upward direction. The sample in the stainless steel vessel was pressed with the plunger for a compression distance of 3 mm.

**Solubilization of the heated ovalbumin sample.** A 0.2 ml sample of 5% (w/v) ovalbumin solution in a test tube was sealed with Parafilm. After being heated at 80°C for 1 hr, the sample was cooled with tap water for 3 hr, and then 1.8 ml of one of the following buffers was added:
- buffer A, 0.1 M sodium phosphate, pH 7.2
- buffer B, buffer A containing 1% (w/v) SDS
- buffer C, buffer A containing 6 M urea
- buffer D, buffer A containing 50 mM 2-mercaptoethanol

The gel was mechanically subdivided using a spatula, and then centrifuged at 3000 x g for 20 min, and the absorbance at 280 nm of the supernatant obtained was measured. The same treatment without heating was done as a control experiment.

**Estimation of the molecular weight by high-performance liquid chromatography (HPLC) in SDS.** A 0.5 mg of 5% (w/v) ovalbumin solution in a test tube was sealed with Parafilm. The tube was heated for 1 hr at 80°C and then cooled with tap water. To each test tube, 9.5 ml of 0.1 M sodium phosphate buffer containing 1% (w/v) SDS, 1 mM EDTA and 2.25 mM N-ethylmaleimide (NEM) was added. The gel was mechanically subdivided and shaken for 15 hr at 37°C to solubilize the gel, before the sample was dialyzed against this buffer without NEM and the dialysate filtered through an Ektrodisc (filter pore size, 0.45 μm). A 100 μl sample of the filtrate obtained was poured over a TSK gel G 4000-SW (7.5 i.d. x 600 mm, Toyo Soda Manufacturing Co., Ltd., Tokyo) that had been equilibrated with 0.2 M sodium phosphate buffer, pH 7.0, containing 0.2% SDS. A Shimadzu high-performance liquid chromatograph (LC-5A) was used. Elution was carried out at the ambient temperature with the same buffer without NEM at a flow rate of 0.3 ml/min. The protein eluted was detected by measuring the absorbance at 280 nm using a Shimadzu SPD-2A UV detector for HPLC and a Shimadzu chromatopac C-R1B. The chart speed was 2 mm/min.

**RESULTS**

**Effect of pH**

The pH profiles of the turbidity and hardness of the heat-induced gel of a 5% (w/v) ovalbumin solution in 20 mM NaCl kept at 80°C for 1 hr are shown in Fig. 1. Between pH 4 and 6, a turbid gel was formed. With a small change in pH, from pH 3.5 to 4.0 or from pH 6.0 to 6.5, the turbidity changed drastically; at around pH 6.5 and 3.5, the gel was slightly turbid, and at more acidic and alkaline pHs, the gels were transparent (see photographs at the bottom of Fig. 1).

Gel hardness had two maxima, at pH 3.5 and 6.5. These were the pHs critical for gel turbidity. The transparent gel prepared at around pH 3.5 and 7.0 was harder than the turbid gel prepared at around pH 5.0, more water being released from the turbid gel.

The conductivity of the solution varied depending on the pH, but not by very much. Thus, in the presence of 20 mM NaCl, firm and transparent gels were obtained in a narrow pH range through which the turbidity of the gel was slightly increasing.

**Effect of NaCl concentration**

The effect was examined of NaCl concentration on the hardness and turbidity at pH
Fig. 1. Hardness and Turbidity of a Heat-induced Gel from Ovalbumin at Various pHs. The 5% (w/v) ovalbumin solution with 20 mM NaCl was heated at 80°C for 1 hr. ○—○, hardness; ■—■, turbidity (absorbance at 600 nm); ×—×, electric conductivity.

Fig. 2. Hardness and Turbidity of a Heated Sample from Ovalbumin at Various NaCl Concentrations. (1) pH 3.5, (2) pH 5.5, (3) pH 7.5. ○—○, hardness; ■—■, turbidity.

At pH 5.5 (Fig. 2-2), all the samples were turbid after being heated. The absence of NaCl gave a turbid suspension containing a coagulum of ovalbumin. In the presence of NaCl, turbid gels were obtained, gel hardness being very low at all salt concentrations.

At pH 3.5 and 7.5 (Figs. 2-1 and 3), in the

3.5, 5.5 and 7.5 (Fig. 2). The appearances of the heated ovalbumin solutions are described at the bottom of the figures.
Fig. 3. Resolubilization of an Ovalbumin Gel Heated at Various NaCl Concentrations.

Ovalbumin solutions were pH 3.5 (1), 5.5 (2), or 7.5 (3). ○—○, buffer A, 0.1 M sodium phosphate, pH 7.2; ●—●, buffer B, 1% (w/v) SDS in buffer A; ×—×, buffer C, 6 M urea in buffer A; △—△, buffer D, 50 mM 2-mercaptoethanol in buffer A.

absence of NaCl or with a low concentrations of NaCl, transparent solutions or a very weak gel that changed to a sol upon agitation were obtained. At fairly low concentrations of NaCl (10 to 30 mM at pH 3.5 and 20 to 50 mM at pH 7.5), the gels were transparent and their hardness increased with the increase in NaCl concentration. When NaCl was in higher concentrations, the turbidity of the gel rapidly increased with the increase in NaCl concentration, and the hardness gradually decreased. Thus, different pHs and NaCl concentrations of the 5% (w/v) ovalbumin solutions gave various results: a transparent solution, transparent gel, turbid gel or turbid suspension. Typical results were a transparent solution at 0 mM NaCl and pH 7.5, a transparent gel at 50 mM NaCl and pH 7.5, a turbid gel at 80 mM NaCl and pH 7.5, and a turbid suspension at 0 mM NaCl and pH 5.5. Various combinations of gel hardness and turbidity resulted from various combinations of pH and NaCl concentration, the hardest gel being obtained at the critical NaCl concentrations or pH that gave a transparent or slightly turbid gel.

Solubilization of gels and coagulums

Gelation by a three-dimensional matrix and coagulation yielding turbidity are caused by protein-protein interactions. The force or forces between ovalbumin molecules to cause the aggregation of the molecules upon heating was then examined. The results when heated samples like those in Fig. 2 were treated with SDS, urea or 2-mercaptoethanol are shown in Fig. 3, with the percentage of protein solubilized from the gel or coagulum given for the four different solvents. Samples were prepared at various NaCl concentrations and three different pHs. The turbidity of the samples when heated is described in the bottom of that figure.

In buffer A, 98%, 68% and 5% of the ovalbumin of a sample heated without NaCl was soluble at pH 7.5, 5.5 and 3.5, respectively. At 20 mM NaCl or above, most of the ovalbumin sample was not solubilized. In buffer B, most of the ovalbumin sample was solubilized and yielded in the supernatant obtained. The SDS broke up the gel structure and solubilized the coagulum. Practically all of each sample was solubilized by SDS.

Buffer C somewhat solubilized transparent gels and sols (Figs. 3-1 and -3), but buffer D had little effect. These results suggest that the molecular interaction caused by heating may be mainly hydrophobic, and that hydrogen and disulfide bonding are less important than in gels of gelatin, agar and soybean protein, all
of which are easily solubilized by a concentrated solution of urea and/or 2-mercaptoethanol.

SDS-HPLC analysis of solubilized gels and coagulums

As already mentioned, 2-mercaptoethanol did not solubilize the ovalbumin gel or coagulum, so disulfide bridges between the ovalbumin molecules were not essential for forming the gel or coagulum. However, the formation of disulfide bonds is probably involved in the first step of coagulation, and heating causes ovalbumin to polymerize by intermolecular sulfhydryl-disulfide exchange to form a network. The molecular weights were studied of ovalbumin solubilized by buffer B using high performance liquid chromatography in SDS (SDS-HPLC). Figure 4 shows the relationship between the molecular weights of the standard proteins and their elution positions; this relationship is linear between molecular weights of 36,000 and 330,000. Ovalbumin that was unheated gave a single peak with a retention time of 64 min at different pHs. Ovalbumin heated for 1 hr at 80°C and pH 3.5 without NaCl gave a trans-

![Fig. 4. Relationship between Molecular Weight and Elution Time of Standard Proteins by High-performance Liquid Chromatography in SDS.](image)

Thyroglobulin (molecular weight, 330,000), ferritin subunit (220,000), phosphorylase b (94,000), catalase subunit (60,000), ovalbumin (45,000), and lactate dehydrogenase (36,000) were used.

![Fig. 5. Elution Profiles of an Ovalbumin Solution Heated at pH 3.5, 5.5 or 7.5, with or without NaCl, after High-performance Liquid Chromatography in SDS.](image)
Table I. Appearance and Molecular Distribution of a Heat-treated Ovalbumin Solution

<table>
<thead>
<tr>
<th>pH</th>
<th>NaCl conc. (mm)</th>
<th>Molecular distribution (%)a</th>
<th>Appearance</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Monomer</td>
<td>Dimer</td>
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a Calculated from SDS-HPLC analysis.

Parent solution, as already described. This sample gave four peaks by SDS-HPLC (Fig. 5-1). These peaks seem to correspond to the monomer, dimer, trimer and tetramer of the ovalbumin molecule, estimated from the standard curve in Fig. 4. Figure 5 also shows the similar elution profiles obtained under different conditions. The relative proportions of each peak for monomer, dimer and trimer + polymer were calculated (Table I). At 80 mM NaCl, the proportion of monomers and dimers decreased, while the trimers and polymers increased. In salt-free buffer at pH 5.5, a peak at about 37 min of elution time appeared (the peak furthest to the left in Fig. 5-3), which seems to correspond to the octamer of the ovalbumin molecule. This peak became larger at 80 mM NaCl (Fig. 5-4). At pH 7.5, several peaks from monomer to octamer were seen; the fraction of the monomer was smaller and that of oligomer was larger than at pH 3.5 or 5.5.

It is thought that the oligomers seen here arose through disulfide bonding between ovalbumin molecules. When the samples were treated with dithiothreitol and then studied by SDS-HPLC, a single peak having the same retention time as that of native ovalbumin was obtained. The addition of 2-mercaptoethanol to the ovalbumin solutions did not inhibit the formation of a heat-induced gel (data not shown). These results show that the disulfide bridge is not primarily of importance in gel formation and that there is no clear relationship between the heat-induced turbidity and gelation of ovalbumin. A giant polymer of ovalbumin with disulfide bridges was not formed by heating the ovalbumin solution, although such heating caused gelation or coagulation of the protein.

DISCUSSION

The profile of gel hardness plotted against pH shown in Fig. 1 is essentially consistent with results reported elsewhere,7,14 where plots of gel rigidity against pH showed two maxima on the acid and alkaline sides of the isoelectric point (pI). Here, it was found quantitatively that not only the hardness but also the turbidity depended on the pH and ionic strength of the medium. In particular, by a change of only 0.5 unit of pH at around pH 3.5 and 6.5, the turbidity of the gel drastically changed. The two maxima of gel hardness at these critical pHs (3.5 and 6.5) gave either a slightly turbid or transparent gel. This might be explained as follows. Each sample contained the same amount of protein. The amount of protein contributing to the formation of the gel network increased as the pH approached the pI. But when the pH was over 3.5 or below 6.5, some of the protein that would have helped form the gel network below pH 3.5 or over 6.5 instead formed a coagulum. Consequently, the number
Firm, Transparent Gels of Ovalbumin

of protein molecules forming the network decreased. For this reason, the turbid gel prepared at around pH 3.5 or 6.5 was weaker than the transparent or slightly turbid gel prepared at a pH closer to the pI. An increase in the NaCl concentration gave similar effects. From these results, we conclude that the electrostatic repulsive force greatly affected the nature of this heat-induced gel. At the critical pH and critical ionic strength just described, the electrostatic force was probably balanced by the attractive force (here, the hydrophobic force) to give an ordered three-dimensional gel network of maximum hardness.

The turbidity and hardness of the heat-induced gel obtained at various pHs and ionic strengths can be explained in terms of their molecular associations as follows. Heating an ovalbumin solution unfolds the native conformation of the molecules and exposes hydrophobic areas to the outside. Then, the hydrophobic interaction originally helping to stabilize the native structure begins to participate in intermolecular associations. In even transparent solutions, soluble aggregates have been found by gel chromatography (in preparation). Foster and Rhees\textsuperscript{15} have also found polymers (molecular weight, 300,000) under similar conditions. When the pH approaches pI, electrostatic repulsive forces become weak and each molecule associates together to form a gel network structure. In the pH region around pI, some of the molecules tightly associate together to form a coagulum. In this state, the intermolecular attractive force might be correlated with the formation of the coagulum. Increased ionic strength in this region of pH lowers the electrostatic attraction and results in the formation of a turbid gel. Such a gel is soft and the water-holding ability of the gel is low, due to the presence of coagulum in the gel network. The surface charge, which acts as a repulsive force, increases with the pH change of medium away from the pI. However, an increase in ionic strength lowers the electrostatic repulsive force by its shielding effect. Balanced hydrophobic interaction and electrorepulsive force would give the best conditions for the formation of gel structures.

When attractive forces are stronger than repulsive forces, a turbid suspension containing a tightly-bound coagulum is formed. When attractive forces decrease, a turbid gel is formed. Coagulum is included in a gel network. As attractive forces decrease much more, a transparent gel is made, and when attractive forces are weaker than repulsive forces, the gel structure is destroyed and a solution containing a soluble aggregate obtained.

Here, transparent, firm gels were obtained with in relatively narrow ranges of pH and ionic strength. This would restrict the practical use of the transparent gel as a food material. Preparation of a hard, transparent gel in wider ranges of pH and ionic strength is being investigated.

REFERENCES