Heat-induced and Transparent Gel Prepared from Hen Egg Ovalbumin in the Presence of Salt by a Two-step Heating Method

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Heat-induced transparent gels from ovalbumin solution can be prepared, but only at low ionic strength, which limits their practical uses. We describe here their preparation at different salt concentrations. Ovalbumin solution (5% w/v) was heated at 80°C, pH 7.0, for 1 hr without salt to give a transparent solution. This solution was cooled and reheated with 0.2 M NaCl, which converted it to a transparent gel. At higher NaCl concentrations, the gel was harder and slightly turbid. We examined the change in the ovalbumin molecule when heated without salt. Gel filtration showed that with heating, the amount of ovalbumin monomers decreased and another peak appeared. After 30 min of heating, there was only one peak. This was a soluble aggregate. SDS-HPLC showed that this soluble aggregate was composed of monomers and oligomers connected by a disulfide bridge or bridges. A conformational change was detected with the difference spectrum. The number of sulfhydryl residues decreased slightly.

The preparation of a firm, transparent gel from egg white ovalbumin by heating under certain conditions has been described elsewhere. Transparent solutions, turbid gels, turbid suspensions, etc., also can be prepared using different pHs and ionic strengths during the heating.

Some proteins form a transparent gel during heating that are different from a heat-induced ovalbumin gel. For example, gels made of gelatin are set by cooling and are heat-reversible, like agar gel and other polysaccharide gels. Reheating of the gel breaks the gel structure. The transparent gel from ovalbumin is set by heat and is heat-irreversible. This makes it possible to heat-sterilize the gel after gelation without deforming its shape.

Other proteins also form heat-setting transparent gels, but usually the conditions such as ionic strength and pH during heating must be carefully controlled. The transparent gel of ovalbumin is not an exception. A certain pH, low ionic strength, or both are needed. To make it possible to use an ovalbumin gel in novel applications for food materials such as a gelling agent, binding agent for chunk-type products, or a meat extender, a method to prepare the transparent gel from ovalbumin even in the presence of salt is necessary. The formation mechanism of various colloidal systems of ovalbumin induced by heating that depends on the medium conditions is of interest.

Here, we found that a transparent gel can be made with high salt concentrations during reheating of a soluble aggregate prepared from an ovalbumin solution by heating in salt-free conditions.

MATERIALS AND METHODS

Ovalbumin and other reagents. Ovalbumin was prepared from fresh egg white as described previously. Purified ovalbumin was dialyzed against distilled water containing 0.05% (w/v) NaN₃ and 0.1 mM EDTA. The pH of the

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dialyzed ovalbumin solution was adjusted to 7.5 by the addition of NaOH. The conductivity of the sample was measured on a conductivity meter (CD-35-M2, M & S Instruments Inc.). This ovalbumin solution was used in the following experiments. Dithiothreitol (DTT), N-ethylmaleimide (NEM), and 5,5'-dithio (2-nitrobenzoic acid) (DTNB) of specific analytical grade were purchased from Nakarai Chemicals, Ltd., Kyoto.

Measurement of SH groups. To 0.5 ml of 5% (w/v) ovalbumin solution that had been heated for a given time, we added 4.5 ml of 0.1 m sodium phosphate buffer, pH 7.0, containing 1% (w/v) SDS and 1 mM EDTA. The ovalbumin concentration of this sample was 5 mg/ml. It was diluted 100 times with 40 mM sodium phosphate buffer, pH 8.0, containing 0.48% SDS and 1 mM EDTA, and incubated for 5 min at 50°C. Then 0.33 ml of 10 mM DTNB solution was added to 5.0 ml of the sample solution, and incubated for 20 min at 50°C. The sample was cooled by tap water and its absorbance was measured at 412 nm. A molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm of 2-nitro-5-mercaptobenzoic acid was used for calculation of the modified sulfhydryl groups in the ovalbumin molecule using DTNB.⁶

High-performance liquid chromatography on TSK-GEL SW in SDS. First, 0.5 ml of ovalbumin sample was added to 9.5 ml of 0.1 m sodium phosphate buffer, pH 7.0, containing 1% (w/v) SDS, 1 mM EDTA, and 0.25 mg/ml NEM, and incubated for 5 hr at the ambient temperature to block the free sulfhydryl groups, preventing disulfide exchange and oxidation. Then the sample was dialyzed against the same buffer without NEM, and filtered with an Eicodisk filter, (pore size, 0.45 μm). The filtrate was put on a TSK gel G 4000-SW (7.5 mm × 600 mm) 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 done at the ambient temperature with the same buffer without NEM. The flow rate was 0.3 ml/min. Protein was detected by absorbance at 280 nm using a Shimadzu UV detector SPD-2A for HPLC and a Shimadzu Chromatopac C-R1B; the chart rate was 2.0 mm/min.

Difference spectrum. First, a 5% (w/v) ovalbumin solution, pH 7.5, without salt was heated and then put on a Toyopearl HW-60 superfine column (1.5 cm i.d. × 100 cm; Toyo Soda Mfg. Co., Ltd., Japan). Elution was done with 20 mM sodium phosphate buffer, pH 7.5, at a flow rate of 0.9 ml/min, and 2-ml fractions were collected. The effluents were monitored by absorbance at 280 nm.

Gel permeation chromatography. Here, 0.5 ml of 5% (w/v) ovalbumin solution, pH 7.5, without salt was heated and then put on a Toyopearl HW-60 superfine column (1.5 cm i.d. × 100 cm; Toyo Soda Mfg. Co., Ltd., Japan). Elution was done with 20 mM sodium phosphate buffer, pH 7.5, at a flow rate of 0.9 ml/min, and 2-ml fractions were collected. The effluents were monitored by absorbance at 280 nm.

RESULTS

Heat treatment

Without heat treatment, the addition of NaCl to an ovalbumin solution (5% w/v, pH 7.5), did not affect its transparency at the ambient temperature, but the heating of ovalbumin solution in the presence of NaCl changed its appearance, turbidity, and hardness, depending on the concentration. When ovalbumin solution was heated in the presence of 50 mM NaCl, a hard, transparent gel was obtained (Fig. 1). When the gel was heated at NaCl concentrations from 50 to 100 mM, the absorbance of the sample increased, indicating turbidity (Figs. 1 and 2A). The hardness of the product decreased with increase in NaCl concentration spectrum of the sample was measured at the ambient temperature against the reference solution of ovalbumin which was not heated. A Shimadzu UV-Visible recording spectrophotometer UV-240 with a Shimadzu graphic printer PR-1 was used. The light path of the sample and reference was 1 cm.

Other methods. Preparation of ovalbumin, adjustment of pH, measurements of protein and NaCl concentrations, turbidity, hardness of the sample, and high-speed gel filtration in SDS aqueous solution on TSK-GEL SW type were done as described previously.¹³

![Fig. 1. Effects of NaCl Concentration on the Hardness and Turbidity of Heat-treated Ovalbumin.](image-url)
Transparent Gel of Ovalbumin in the Presence of Salt

Fig. 2. Effects of NaCl Concentration and First Heating Period on the Appearance of Heated and Reheated Ovalbumin Solutions.

The ovalbumin solution (5% w/v, pH 7.5) was heated for 1 hr at 80°C in the presence of NaCl ranging in 0 to 0.5 M (A). Ovalbumin heated already in the absence of NaCl was reheated at 80°C for 1 hr at various NaCl concentrations (B), or for various heating periods at 80°C and 0.2 M NaCl (C).

Increasing NaCl concentration in this range of concentrations. Above 100 mM NaCl, all samples were turbid and the hardness of the gel increased slightly up to about 300 mM.

Reheating treatment

In the absence of salt, heating of ovalbumin solution (5% w/v, pH 7.5) gave neither gelation nor turbidity. When the ovalbumin solution was heated without salt, and then heated again after the addition of NaCl, a gel was obtained the hardness of which depended on the NaCl concentration (Figs. 2B and 3). However, the turbidity did not increase as much as compared to that in Figs. 1 and 2A. This shows that at high salt concentrations, a transparent heat-induced gel could be made from an ovalbumin solution. The transparent gel prepared by reheating with 300 mM NaCl was harder than the turbid gel prepared by heating at 300 mM in Fig. 1. The first salt-free heating (pre-heating) was necessary to obtain a transparent gel by reheating in the presence of NaCl. The conditions of the first heating were examined next.

Effects of time of first heating on gel turbidity

Salt-free ovalbumin solution (5% w/v, pH 7.5) was heated at 80°C for various times up to 60 min; after being cooled, 0.2 mM NaCl was added. The absorbance of each sample was measured (open circles in Fig. 4). When the first heating was for 20 min or more, the samples were transparent. Their absorbances were below 0.1.

All of these samples containing 0.2 mM NaCl were reheated at 80°C for 1 hr. The absorbances of these samples after being cooled were
FIG. 4. Effects of First Heating Time on Turbidity.

Some 2.0 ml of the ovalbumin solution (5% w/v, pH 7.5) was heated at 80°C for various periods in the absence of NaCl, and after the sample was cooled in tap water for 1 hr, 0.5 ml of 0.2 M NaCl solution was added. The absorbance at 600 nm was measured (○). Then each sample was heated again at 80°C for 1 hr. After it was cooled, the absorbance at 600 nm was read again (■). The absorbances are plotted against the first heating period.

plotted against the time of first heating by the closed circles in Fig. 4. When the sample was heated for 2 or 3 min in the absence of NaCl, its absorbance for the reheated sample in the presence of NaCl was 0.90 or 0.36, respectively. By a longer first heating, the absorbance of the reheated sample, decreased to below 0.2, indicating that a transparent gel was obtained by reheating in the presence of 0.2 M NaCl (Figs. 2C and 4).

The temperature at the center of the sample tube was monitored using a thermister (insert in Fig. 3). The temperature of the sample reached 80°C after 1 min of heating, when the solution had already changed to a gel.

These results mean that a transparent gel could be made from ovalbumin solution not only by heating when salt-free, but also by a second heating in the presence of salt when first heated in the absence of salt for several minutes at 80°C. This shows that some molecular change in the ovalbumin occurred during

the first salt-free heating that lowered the sensitivity to NaCl. This molecular change was studied in the following experiments.

Formation of soluble aggregate during first heating

Gel filtration of ovalbumin heated while salt-free was done using a Toyopearl HW-60 column (Fig. 5). Native ovalbumin (5% w/v) gave a single peak (Peak A; elution volume, 96 ml). When heated at 80°C, the ovalbumin gave a new peak (Peak B; elution volume, 76 ml) of higher molecular weight, and the height of this new peak increased with the heating time. With further heating (first heat-
ing, 30 min; Fig. 5), peak A disappeared and only peak B remained. Since peak B was a symmetrical curve and no other peaks or shoulders were found, the heat-induced soluble aggregate obtained in salt-free conditions seem to have a narrow distribution of molecular weight.

High-performance liquid chromatography (HPLC) in SDS

To discover the force supporting the molecular interaction of the formation of the polymer, we looked for evidence of covalent bonds (disulfide bridges) between ovalbumin molecules. The transparent solution obtained by first heating in the absence of salt was analyzed with SDS-HPLC using a TSK gel G 4000-SW column in SDS-sodium phosphate buffer (Fig. 6). When native ovalbumin was used, a single peak with the retention time of 40 min was obtained (insert in Fig. 6). As the time of the first heating increased, other peaks corresponding to polymers of higher molecular weight appeared and their height increased. Heating for 2 min gave four different peaks, and the original peak of native ovalbumin became smaller. When the molecular weights were calculated from a standard curve obtained using authentic proteins (thyroglobulin subunit, 330,000; ferritin subunit, 220,000; phosphorylase b, 94,000), each peak at 2 min in the insert in Fig. 6 corresponded to monomers, dimers, or trimers of the ovalbumin molecule. Heating for a long time (30 or 60 min) gave a new peak at the elution position.

![Fig. 6. Elution Profiles by High-performance Liquid Chromatography on TSK-GEL SW in SDS of Ovalbumin Heated without Salt for Various Periods, and the Change of the Molecular Distribution in the Sample with Heating.](image)

![Fig. 7. Changes in the Difference Absorption at 257 nm between Native Ovalbumin and Ovalbumin Heated When Salt-free, pH 7.5, and the Changes in the Sulfhydryl Content and the Monomer Fraction in Ovalbumin upon Heating.](image)
of 32 ml, whose molecular weight was, however, smaller than that of the thyroglobulin subunit. That is, long heating did not give a polymer with a large molecular weight through disulfide bridges. The molecular fractions of monomers, dimers, trimers, and tetramers in the sample heated were plotted against the heating time (Fig. 6). Monomers decreased with increasing the heating time, accompanied by an increase in dimers, trimers, and tetramers. After 30 min, tetramers were predominant in the mixture.

Formation of disulfide bonds
To confirm that the oligomers were formed through disulfide bridges between ovalbumin molecules, dithiothreitol (100 mM) was added to native ovalbumin solution (5% w/v, pH 7.5), and the mixture was heated for 30 min at 80°C. The heated sample obtained was analyzed with SDS-HPLC as described above. One single peak was obtained at the same elution position as that of the native ovalbumin; there were no other peaks.

The ovalbumin molecule has four free sulfhydryl residues. We measured the number of sulfhydryl groups of ovalbumin molecules heated for various times to know whether the formation of a polymer was caused by the oxidation of cysteine residues between two molecules or by disulfide interchange between them. The reduction in the numbers of SH groups was not large (Fig. 7). With 30 min of heating, the decrease was only about 0.2 mol/mol of ovalbumin. This is not enough to form oligomers by oxidative polymerization. Therefore, the disulfide bridge resulting in the oligomer in Fig. 6 seems to be formed by an interchange reaction of cysteine residues in the ovalbumin molecule.

The aggregate observed in Fig. 6 was much smaller than that shown in Fig. 5. This suggests that the major intermolecular bond that formed the soluble aggregate was a noncovalent one: a hydrophobic, hydrogen, or electrostatic bond, or another. The formation of a soluble aggregate and oligomer reached a constant state within 30 min of heating, as stated above. This molecular association seems to be accompanied by a conformational change of the ovalbumin molecule with heat.

Conformational change of ovalbumin
The difference spectra of the ovalbumin solutions heated for various times against native ovalbumin solution are shown in Fig. 8. A difference in absorbance was observed in the UV region after 1 min of heating. This difference absorption increased with further heating. The difference absorption at 257 nm was plotted against the heating time in Fig. 7, where the change in the number of monomers and sulfhydryls proceeded almost simultaneously. The time dependency of the reduction in num-

Fig. 8. Difference Spectrum of Ovalbumin Heated without NaCl against Native Ovalbumin. The heating time is indicated in the figure.
TABLE I. EFFECTS OF COOLING TIME AFTER HEAT TREATMENT ON TRANSPARENCY, NUMBERS OF SULFHYDRYL GROUPS, AND MOLECULAR PATTERN OF OVALBUMIN*  

<table>
<thead>
<tr>
<th>Cooling time (hr)</th>
<th>Turbidity $^b$</th>
<th>SH groups mol/mol of ovalbumin</th>
<th>Molecular pattern$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.148</td>
<td>3.98</td>
<td>26.0</td>
</tr>
<tr>
<td>3</td>
<td>0.143</td>
<td>3.86</td>
<td>N.A.$^d$</td>
</tr>
<tr>
<td>6</td>
<td>0.220</td>
<td>3.90</td>
<td>23.2</td>
</tr>
<tr>
<td>24</td>
<td>0.169</td>
<td>3.89</td>
<td>22.3</td>
</tr>
</tbody>
</table>

$^a$ 5% (w/v) ovalbumin solution, pH 7.5, was heated for 1 hr at 80°C. The heated ovalbumin was cooled in tap water for the time indicated in the table. After cooling, turbidity, the number of SH groups, and the molecular pattern were analyzed.

$^b$ Absorbance at 600 nm using a cell with a 1-cm light path.

$^c$ Calculated from the SDS-HPLC pattern of each sample. The measurement is the same as in Fig. 7.

$^d$ Not assayed.

When ovalbumin is heated, denaturation and aggregation proceed continuously. In the presence of salt at about 0.1 M, some parts of the molecules. The aggregate seems to be composed of ovalbumin molecules associated by hydrophobic or other noncovalent bonding as the core of the oligomer linked with a disulfide bridge. This soluble aggregate forms a gel network without coagulation under the high ionic conditions with reheating.

The effects of cooling time after the reheating treatment are shown in Table I. Transparency, numbers of sulfhydryl groups, and molecular pattern in the sample did not change. The formation of new disulfide bridges does not seem to occur by oxidation.

DISCUSSION

The heat-induced aggregation of protein molecules follows a two-step process: denaturation and aggregation. The latter strongly depends on the environment. The two-step process is as follows:

1. Native protein → Denatured protein → Aggregate
2. Coagulum → Protein network → Soluble aggregate

Increase in Salt conc.
coagulum. The network and coagulum give the rigidity and turbidity, respectively, of the sample.\(^1\) A turbid gel seems to comprise a network structure and coagulum, and a transparent gel seems to comprise a protein network but does not appear to contain coagulum. However, in the absence of salt, the aggregation step is repressed by electrostatic repulsion between denatured protein and a soluble aggregate consisting of oligomers. We assume that this aggregate takes the form of an ordered linear polymer. Transmission electron microscopic studies of the soluble aggregate gave filamentous images, which indicated the linear aggregation of molecules (unpublished data). After the soluble aggregate is cooled and NaCl is added, this transparent solution changes to transparent gel. Reheating activates the ovalbumin molecules that make up the soluble aggregate, and then this linear aggregate associates to form a gel network. The soluble aggregate might be maintained in the network structure as a constituent unit, because if the soluble aggregate dissociates to monomers during heating in the presence of NaCl, the monomers would associate randomly to form a coagulum and result in a turbid gel. This means that the balance of attractive and repulsive forces among the denatured molecules is different from that of molecules in a soluble aggregate, and the soluble aggregate behaves as an association unit for a larger formation, namely, a gel network structure.

This paper showed that first heating of ovalbumin without salt gave a soluble aggregate, which in turn became a transparent gel in the presence of salt upon reheating. A molecular change in the ovalbumin denatured under low salt concentrations was seen.

REFERENCES