Melting of the Ovalbumin Gels by Heating: Reversibility between Gel and Sol

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Heat-induced ovalbumin gels prepared under given conditions were melted with a second heating to below 90°C. The sample melted was gelled again on cooling. This gel-sol transition, which was detected both by eye and by the change in the dynamic viscoelastic parameters $G'$ and $G''$, was reversible. Differential scanning calorimetry showed that the endothermic heat flow that accompanied the gel-sol transition was much lower than that accompanied the denaturation of ovalbumin. With the repetition of the gel-sol transition, intermolecular disulfide bridges were formed, detected by sodium dodecyl sulfate-polyacrylamide electrophoresis done without a reducing agent.

Egg white is a popular food and is not only eaten with little processing, but also used in a variety of foodstuffs because it has various functional properties[1]. The mechanism of the expression of the functional properties of egg white protein and their relation to thermal denaturation of protein have been studied[2,3]. The gelling property of egg white protein has been investigated in detail[4-6]. Ovalbumin, the major protein of egg white, forms a turbid gel when heated at a high protein concentration. Ovalbumin molecules denatured by heat interact to give aggregates with various molecular forces. Hydrophobic interaction among the denatured ovalbumin molecules is the predominant attractive force for aggregation, and electrostatic force causes repulsion among the denatured molecules[7,8]. When the attractive forces are much stronger than the repulsive forces, the molecules aggregate randomly, because one molecule can bind with several other molecules and give a large coagulum that results in a turbid gel or suspension. A large random aggregates of denatured ovalbumin molecules were observed by electron microscopy. When the interaction is restricted by repulsive forces, the number of contacts between the molecules is probably reduced[9,10]. Molecules polymerized with “head and tail” conjunctions gave linear aggregates, gel network, and a transparent gel structure[11]. When both linear and random aggregates exist, the gel becomes turbid. Similarly, turbid and transparent gels can be prepared from egg white by control of the pH and ionic strength of the sample[12,13].

In a transparent gel, the protein molecules seem to bind together weakly, because the hydrophobic attractive and electrostatic repulsive forces are balanced. When the net forces acting between denatured molecules are weak, hydrogen bonds that might form between denatured molecules become more important. If such interaction contributes to network formation, the gel structure may change and the gel may melt when heated because hydrogen bonds become weaker at higher temperature, unlike hydrophobic interactions. Here, we undertook the melting of transparent gels to know the forces contributing the network formation of denatured ovalbumin molecules.
Materials and Methods

Preparation and heat treatment of ovalbumin solution

The ovalbumin used in this study was purified from fresh egg white by repeated crystallization from ammonium sulfate as described before. Crystallization was done five times. The concentration of ovalbumin was calculated from the absorbance at 280 nm based on the value of E = 7.12. The purity of the ovalbumin was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli. Before heat treatment, the ovalbumin solution was exhaustively dialyzed against 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 0.02% NaN₃, and 20 mM NaCl at 4°C. The precipitate that appeared after dialysis was removed by centrifugation and the pH of the supernatant was adjusted to 7.0 by the addition of a small amount of NaOH solution, if necessary. Preparation of gels by heating was done as follows. One milliliter of ovalbumin solution (37.5 mg/ml) was put in a 1-ml transparent-glass vial closed with a stainless steel ball (diameter, 5.0 mm) placed on the mouth (i.d., 4.8 mm) of the vial. The vial containing the ovalbumin solution was placed in a water-bath at 80°C for 20 min and cooled with tap water for 1 hr.

Measurement of the melting temperature of gels

A stainless steel ball (diameter, 4.0 mm) was put on the gel prepared as described above and then the glass vial was tightly sealed with aluminum cap. The vial was placed in a water-bath in a transparent vessel and heated at the programmed heating rate of 3°C/min until 90°C was reached. Changes in the fluidity of the gel were directly observed from outside of the vessel by the movement of the stainless steel ball, which was put on the gel. The temperature at which the ball reached the bottom of the vial was taken to be the melting temperature of the gel.

Differential scanning calorimetry

Thermal denaturation of ovalbumin was studied by differential scanning calorimetry with the model DSC 100 calorimetry connected to a SSC 5000 (Seiko Instruments Inc., Tokyo). The sample (50 μl, 37.5 mg/ml) was put into a silver pan. First, the sample was heated to 80°C at the programmed rate of 3°C/min and kept at 80°C for 20 min. Then the sample was cooled down to 30°C at the rate of 3°C/min, heated again at the same heating rate up to 90°C, and cooled again to 30°C at the rate of 3°C/min.

Measurement of mechanical properties

The gelling behavior and melting of the gel were monitored with a device for the measurement of dynamic viscoelasticity (Rheographsol, Toyoseiki, Inc., Tokyo). The ovalbumin solution (20 mg/ml, 1.6 ml) was poured into a clam-shell shaped cell and heated at the programmed rate of 3°C/min from 23°C to 80°C. The sample was kept for 20 min at 80°C and then cooled to 30°C at the programmed rate of 1°C/min. The cooled sample was heated again to 90°C and then immediately cooled to 30°C at the same rate. Humidified air was blown through the chamber to prevent evaporation from the sample during testing and the cell was capped with a teflon cover. The storage modulus, G', and loss modulus, G", of the sample material were measured.

Transmission electron microscopy (TEM)

Ultrathin sections of the gels were observed by TEM as follows. The gel was fixed with 2.5% glutaraldehyde and 2% osmium tetroxide, and then dehydrated with ethyl alcohol. The dehydrated gel was embedded in Luveak-812 (Nacalai Tesque, Kyoto) to make a block. After being trimmed, the sample was sectioned with a microtome. The thin piece was put on a support mesh and stained with uranyl acetate (4% w/v) and lead acetate (4% w/v). The sample obtained was observed with a transmission electron microscope (Hitachi, H-700 H) at 100 kV.

Results

Melting of gels

When the ovalbumin sample was heated and then cooled, a transparent gel was formed.
When the gel was heated, the stainless steel ball put on the gel fell to the bottom of the vessel at 82°C, and the sample gelled again after being heated and cooled (Fig. 1), showing that this heat-set transparent ovalbumin gel melted when heated to below 100°C, and reversibly gelled again when cooled. That is, gel-sol transition with heating and cooling, which has been observed in the cold-set gelation of gelatin and agarose, occurred in an ovalbumin gel. Until now, it has been believed that egg white and ovalbumin gels were heat-set gels and that a gel once formed could not be melted by being heated again to below 100°C; no reports have mentioned the reversibility of the gel-sol transition of an egg white or ovalbumin gel.

When heating and cooling of the same sample was done a total of six times, the sample remained transparent and the gel-sol transition occurred each time. On the second heating, the melting temperature increased by 15°C, and on the third heating, the melting temperature became 104°C; the fourth, fifth, and sixth heatings gave a melting temperature in this vicinity (Fig. 2). To know the changes in the ovalbumin molecules during heating and cooling, the sample in each heating step was examined by SDS-PAGE. The ovalbumin samples after the first or second heating gave a single band of ovalbumin on SDS-PAGE with 2-mercaptoethanol (Fig. 3). Without 2-mercaptoethanol, the samples gave the other bands with higher molecular weight than that of the original ovalbumin. Some species could not enter the separation gel of polyacrylamide gel because of high molecular weight. On the third and fourth heating, the sample gave a similar pattern of SDS-PAGE with and without 2-mercaptoethanol. This means that the repeated heating and cooling did not change
the primary structure of the ovalbumin molecules except the formation of intermolecular disulfide linkages. From these results, the increase in the melting temperature after repeated heating and cooling seemed to be caused by the formation of covalent bonds by intra- and intermolecular disulfide bridges during these steps. However, this molecular change had little effect on the reversibility of the gel–sol transition.

Differential scanning calorimetry

Melting of gels is usually endothermic. This change can be detected by DSC measurement. Fig. 4 shows the DSC patterns in the first, second, and third heating of ovalbumin samples. The samples had the same protein concentration as those used in the above experiments, and the rate of increase in the temperature and the temperature profile were also the same. A large endothermic peak at 78.7°C (peak top) was found at the first heating. This peak corresponds to the denaturation of the ovalbumin molecules. In the second and third heating, endothermic heat flow was not observed, although the gel that formed in the DSC pan was melted at these times, to judge from the results above. This indicates that the thermal change that accompanies the gel–sol transition was much less than the thermal change that accompanied the denaturation of ovalbumin. Therefore, the gel network seemed to be formed by weak molecular interactions between denatured and the formation and breakage of such linkages might not be detected by DSC measurement.

Dynamic viscoelastic behavior

Gel–sol transitions were directly observed by eye in the experiment above. To measure this transition quantitatively, the changes in the dynamic viscoelastic parameters of the ovalbumin sample with heating and cooling were measured. Fig. 5 shows the profiles of the temperature, the storage modulus, G’, and the loss modulus, G”, with the repetition of heating and cooling. From 23°C to 80°C, G’ and G” changed little, but when the sample was kept at 80°C for 20 min, both G’ and G”

Fig. 4 DSC thermograms of ovalbumin during gel–sol transitions
(1), first heating; (2), second heating; and (3), third heating.

Fig. 5 Changes in the storage modulus, G’, and the loss modulus, G”, of an ovalbumin sample during heating and cooling
The protein concentration of the sample was 37.5 mg/ml. The heating and cooling rate were 3°C/min and 1°C/min, respectively.
increased, corresponding to the gelation of the ovalbumin. With decreases in the sample temperature from 80°C to 30°C, G' and G" increased more, reaching a maximum. Then the values lowered steeply during the second heating from 30°C to 90°C. This indicates that the gel melted, which corresponds to the gel-sol transition observed above. The G' and G" values at 90°C were higher than those of the native ovalbumin solution, indicating that the ovalbumin sample at 90°C was a sol. The G' and G" increased again during the second cooling. The G' and G" in Fig. 5 were slightly higher than these final values of G' and G" obtained by the first cooling. This seems to be due to the difference in the size of the aggregates of denatured ovalbumin. Next, the aggregates forming the gel were directly observed by TEM.

Transmission electron microscopy

The images of the gel by TEM showed the formation of linear aggregates by heat (Fig. 6). By the first heating and cooling, a great number of linear aggregates were formed, and samples heated again gave similar linear aggregates that were longer than those observed after the first heating.

Discussion

On the first heating, the ovalbumin molecules denatured at around 80°C, as seen by DSC, and increases in G' and G" were observed at 80°C. In both cases of DSC and viscodynamic measurements, the sample and heating conditions, including the rate at which the temperature increased, were the same, therefore these results mean that the heat denaturation of ovalbumin corresponds to the heat gelation or network formation by ovalbumin molecules. The heat-set gel of ovalbumin was melted by the second heating and gelled again when cooled. This behavior is that of a cold-set gel. A solution of globular proteins usually gives a heat-set gel, which is not melted by heating of the gel\(^{16}\). However, arachin and milk whey protein isolate can give heat-reversible gels, with a gel-sol transition\(^{17)}^{18)}

It is reported that thermal-coagulation of egg white solution was repressed if the coagulum formed by foaming treatment was removed in advance\(^{19)}

Fig. 6 Transmission electron microscopy images of thin-sectioned ovalbumin gels

(1) gel obtained after the first heating and cooling, (2) gel obtained after the second heating and cooling.

Magnification, × 40,000.
rameters\textsuperscript{22}, and in this study, a heat–set ovalbumin gel melted. If only the hydrophobic interactions, which give rise to attractive forces between denatured molecules, were reinforced with increases in the temperature, the ovalbumin gel would be hardened by heat. The gel network of gelatin or agarose is formed mainly by hydrogen bonds, which are weakened at higher temperatures, and such a gel is melted by being heated. In fact, the ovalbumin gel prepared in this study was weakened and melted by heat, so it seems that hydrogen bonds are an attractive force that helps to form the gel network in addition to the hydrophobic interactions also involved. That is, hydrophobic interactions are the major attractive forces linking the denatured molecules together. Depending on the heating conditions, protein concentration, ionic strength, and pH, the role of the hydrophobic interactions as an attractive force may be repressed and hydrogen bonding may come to be more important. If so, the gel might be weakened and melted when heated. Under conditions of low ionic strength, the ovalbumin gels melt, but ovalbumin gels prepared at high ionic strength or high protein concentrations were not melted when heated at below 100°C. At high ionic strength, the repulsive forces arising from electrostatic interactions are repressed and the attractive forces caused by hydrophobic interaction increase greatly, hence the denatured ovalbumin molecules interact strongly and the gel did not melt at below 100°C.

It has been reported that intermolecular disulfide bridges were formed between ovalbumin molecules during heating\textsuperscript{3,12}. Here, we found that disulfide bridge formation did not affect the gel–sol transition, but the melting temperature increased with the formation of disulfide bridges. In whey protein isolate, the increased gelation rate with each heating cycle may result from increased numbers of disulfide linkages\textsuperscript{18}.

Repeated heating and cooling increased the values of $G'$ and $G''$, and TEM images of the gel showed that repeated heating and cooling made the linear aggregates longer. Therefore, repeated heating and cooling might cause the polymerization of denatured ovalbumin molecules by disulfide bridges, and the longer linear aggregates that resulted would increase $G'$ and $G''$, reflecting the formation of a more elastic gel. However, in this study, the gel–sol transition was observed macroscopically throughout the repeated heating and cooling.

Here, gel–sol transitions in ovalbumin gels were observed when suitable conditions were selected. This gel–sol transition might not be special to ovalbumin since similar results have been found in the gel of other globular proteins\textsuperscript{17,18}. This property of ovalbumin gel could make possible new practical applications of egg white and suggest a different approach to the study of the mechanisms of protein gelation.

References


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卵白アルブミンゲルの加熱融解：ゲル・ソル間の可逆性
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一定条件下で加熱調製して得られた卵白アルブミンゲルは、90℃以下の加熱によって融解し、冷却に伴って再びゲル化した。このソル-ゲル転移は可逆的であった。この変化は目視観察、および動的粘弹性パラメータにより調べ、示差走査熱量計により、ゲル化-ソル転移に伴う吸熱は、卵白アルブミンの熱変性の場合よりもはるかに低いことが示された。ゲル化-ソル転移を繰り返すことにより分子間のS-S架橋が生成され、分子重合体の生成が認められた。