Heating of an Ovalbumin Solution at Neutral pH and High Temperature

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The thermal denaturation, aggregation, and degradation of hen egg white ovalbumin dissolved in distilled and deionized water (60 mg/ml, pH 7.5) was investigated by differential scanning calorimetry (DSC), polyacrylamide gel electrophoresis (PAGE), and viscosity measurement. Two independent endothermic peaks were observed up to 180°C by the DSC analysis. The first peak appeared at around 80°C, corresponding to the denaturation temperature of ovalbumin. The second peak occurred around 140°C due to the degradation of protein molecules as judged from the analysis by SDS-PAGE. The viscosity of the ovalbumin solution increased dramatically above 88°C and maintained almost the same value up until heating to 140°C. The increase in viscosity after heating to 88°C was due to the denaturation and subsequent aggregation of ovalbumin molecules as observed by SDS-PAGE. The decrease in viscosity of the samples heated above 150°C appears to have been the result of degradation of the ovalbumin molecules.

Key words: egg white protein; ovalbumin; molecular aggregation; degradation; high-temperature heating

Hen-egg white is widely used as an ingredient by the food industry to enhance and improve the functionality of various food products. Ovalbumin (OVA), the major protein of egg white, is a globular protein with a molecular weight of 43,000 and consisting of 385 amino acid residues. It contains four cysteine residues and one cystine residue.1,2) There have been many reports on heating to temperatures less than 100°C to analyze the functional properties of OVA.3) Heating above 100°C in an enclosed moist system is a versatile technique for sterilization in modern food processing. The effects of high temperature heating on the structure and stability of protein molecules has been investigated on thermo-resistant enzymes from thermophilic bacteria. Throughout these studies, deamidation, isomerisation, decomposing of some amino acid residues, and hydrolysis of peptide bonds have been nominated to yield a loss in the functional properties of protein by heating.4) The authors have recently reported the denaturation, aggregation, and degradation of β-lactoglobulin molecules by heating up to 180°C in an enclosed moist system.5)

However, there have not been many reports on the effects of heating other food proteins, including egg white protein, to temperatures above 100°C in an enclosed moist system on their functional properties as food ingredients and the changes at the molecular level.

This study was undertaken with purified ovalbumin to investigate the molecular behavior at high temperature and the relationship with its food functionality in order to provide an approach for use in food processing and to understand the effects of these molecular changes on the quality of food products.

Materials and Methods

Preparation OVA sample and heating. The OVA sample used in the present study was purified from hen-egg white by 6-times crystallization from an ammonium sulfate solution.7) The obtained OVA was exhaustively dialyzed against distilled and deionized water. The OVA dialysate in distilled and deionized water was adjusted to pH 7.5 by diluted NaOH and filtered through a 0.45-μm membrane (Millipore Co., Bedford, MA, U.S.A). The OVA obtained gave a single band by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Heated samples were prepared by the same method as that described elsewhere.5) Briefly, 1.2 ml of an OVA solution (10 mg/ml, pH 7.5) was transferred into glass vials (3.2 cm height; 1.0 cm diameter). Each glass vial was tightly closed with an aluminum cap and heated up to 180°C in a temperature-regulated oil bath at a rate of 6°C/min. All chemicals used were of analytical grade.

Determination of the protein concentration. The concentrations of protein were determined by the method of Lowry et al.8) using bovine serum albumin as a standard.

Materials

The materials used in this study were the following: ovalbumin (OVA), β-lactoglobulin, bovine serum albumin (BSA), and distilled and deionized water.

References


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Abbreviations: OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry
OVA protein concentration was determined spectrophotometrically at 280 nm based on E1% = 7.12.²)

Differential scanning calorimetry (DSC). The thermal behavior of the OVA sample was measured by means of differential scanning calorimetry (DSC) with a DSC 100 calorimeter (Seiko Instruments Co., Chiba, Japan). All experiments were conducted by the following method except where noted. The final concentration of OVA was 60 mg/ml and the pH value was 7.5. A hermetic silver pan (No. 560–003; Seiko Instruments) was pretreated before use as described before.³) Fifty µl of the sample solution was poured into the pan, while 50 µl of distilled and deionized water was used in a reference pan. Heating was performed at the rate of 6°C/min from 25°C to 180°C, the data sampling time interval was 0.5 s, and indium was used to calibrate the instrument. All parameters were computed from the thermogram by DSC analysis software (Seiko Electric Industry Co., Tokyo, Japan).

Gel electrophoresis. Polyacrylamide gel electrophoresis without a denaturant (native-PAGE) was conducted according to the procedure of Davis⁴) on 8% and 4.5% polyacrylamide in separating gel and stacking gel, respectively. The protein solution (0.25 mg/ml) was mixed with an equal volume of glycerol (40% v/v), the sample (2.5 µg) then being loaded into each well of the stacking gel. The protein in a gel were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% methanol.

SDS-PAGE was performed by the method of Laemmli⁵) on 10.0% separating gel and 3.0% stacking gel. A protein sample with or without 2-mercaptoethanol (2-ME) was heated at 100°C for 3 min and then cooled to room temperature. The protein sample (2.5 µg) was applied to each lane.

Tris-Tricine polyacrylamide gel electrophoresis was conducted according to the method of Coligan et al.⁶) with a slight modification. The polyacrylamide concentration of the separating gel and stacking gel were 13.5% and 3.87%, respectively. Unheated and heated OVA samples were diluted to 0.5 mg/ml and mixed with an SDS sample buffer in the presence of dithiothreitol (DTT) at a ratio of 1:1 (v/v), before being incubated at 40°C for 30 min. The final concentration of DTT was 0.2 m. Five µg of protein was loaded into each well of the stacking gel. Tris-HCl (0.2 M, pH 8.9) was used as the anode buffer, and Tris (0.1 M) and Tricine (0.1 M) containing 0.1% (w/v) SDS was used as the cathode buffer. The power supply was 50 V (constant voltage) over about 10 min followed by 150 V (constant voltage). The electrophoresis gel was stained by the same procedure as that used for native-PAGE and SDS-PAGE.

DSC Thermograms of Ovalbumin (OVA) in the Temperature Range of 25°C–180°C at a Rate of 6°C/min.

The pH value and protein concentration of the OVA solution were 7.5 and 60 mg/ml, respectively. i) OVA was heated from 25–180°C. ii) OVA (new sample, 60 mg/ml, pH 7.5) was heated from 25–110°C. iii) The sample in ii) was cooled to 25°C and heated again to 180°C. iv) After cooling, the sample in iii) was heated again to 180°C.

Viscosity measurement. The viscosity was measured immediately after cooling the heated samples. Glass vials containing the OVA solution (10 mg/ml and pH 7.5) were heated in an oil bath at a rate of 6°C/min and then cooled to ambient temperature. One ml of the sample was poured into the cone vessel of a cone-plate type of rotational viscometer (Model E, Tokyo Keiki Co. Ltd., Tokyo, Japan) with a cone plate (24 mm radius and 1°34’ cone angle). The solution was incubated for 3 min at 25.0±0.2°C and maintained at the same temperature with cone jacket in which water at a constant temperature was circulated. The shear rate was changed from 9.6 s⁻¹ to 383.0 s⁻¹. In this study, the apparent viscosity was measured at shear rates of 76.6, 191.5 and 383.0 s⁻¹.

Results

All heated OVA samples were transparent during heating to 180°C and cooling to room temperature at pH 7.5. However, the sample was changed to a clear viscous liquid by heating above 90°C. To clarify the molecular events occurring in OVA by heating, a thermal analysis of the sample was performed first by using differential scanning calorimetry (DSC).

Differential scanning calorimetry (DSC)

The DSC thermogram showed two transition peaks (Fig. 1-i). The onset and the peak top temperatures of the first peak were 72.0±0.5°C and 85.0±0.5°C, respectively. The enthalpy value calculated from the area of the first peak was 8.6 mJ/mg of protein. The onset and the peak top temperatures
of the second peak were 112.0 ± 0.5°C and 137.0 ± 0.5°C, respectively. The enthalpy value calculated from the area of the second peak was 10.4 mJ/mg-protein. To clarify the relationship between these endothermic peaks and the events that had occurred, the native OVA solution was heated from 25°C to 110°C (Fig. 1-ii), cooled to 25°C, and then heated again to 180°C (Fig. 1-iii). The OVA solution developed a peak at around 80°C when first heated (Fig. 1-ii), the second heating produced no peak at around 80°C, and another peak appeared at around 140°C, which was slightly higher than that in Fig. 1-ii (Fig. 1-iii). After cooling to 25°C, the same sample was heated again to 180°C (Fig. 1-iv). No peak was apparent up to 180°C (Fig. 1-iv). These results show that the two endothermic peaks were independent and represented different reactions that occurred in the protein molecules when heated to 180°C; both types of reaction were apparently irreversible over the time frame of the experiment. The endothermic peak at around 80°C corresponds to the thermal denaturation of OVA. The second thermal transition would have been due to another endothermic reaction. To clarify the first and second reactions the OVA solution was heated to various temperatures and then analyzed by native-PAGE and SDS-PAGE.

Analysis by polyacrylamide gel electrophoresis (native-PAGE, SDS-PAGE, and Tris-Tricine SDS-PAGE)

Heating was performed by immersing the glass vial, containing 1.2 ml of the OVA solution (10 mg/ml, pH 7.5) into the oil bath and heating at the same rate of 6°C/min as that used for DSC. The vial was heated to one of several temperatures, and then the heated OVA was analyzed by native-PAGE (Fig. 2). Native OVA gave 3 bands due to the degree of phosphorylation of the molecules, corresponding to A1, A2, and A3 OVA. Heating OVA to 40°C–60°C provided 3 bands with the same result as that of the unheated sample. The band of the monomer was pale after heating to 88°C, and this heated sample gave several bands on the separating gel with lower mobility than that of the OVA monomer. The intensity and number of bands with lower mobility increased with increasing temperature, 93–128°C, while the monomer band was hardly apparent. Several bands on the separating gel of the samples heated to between 88°C and 128°C indicate that the heated OVA molecules had formed large molecular aggregates. When heated to 140°C–150°C, the large molecular aggregates, that is, the bands close to the stacking gel, became pale, while several bands with low mobility were still observed. A faint smear was seen on the separating gel in the case of the samples heated to 160°C, and especially to 180°C.

Heated OVA was analyzed by SDS-PAGE with and without 2-ME (Fig. 3). SDS-PAGE with 2-ME showed a single and sharp condensation of the OVA monomer in the unheated sample and the sample heated to 140°C (Fig. 3; left). The OVA samples heated to 150°C–180°C provided some bands with higher mobility than that of the monomeric band. The SDS-PAGE pattern without 2-ME (Fig. 3; right) reveals that the OVA molecules had aggregated and been polymerized by heating the temperatures higher than 88°C. Referring to SDS-PAGE in the presence of 2-ME in Fig. 3 (left), these molecular aggregates were formed through an intermolecular disulfide covalent linkage. The bands with lower mobility than that of the monomer were pale smears on the separating gel at 160°C and 180°C, and high-mobility, faint bands in the sample heated to 160–180°C were seen similarly to the case of SDS-PAGE with 2-ME. These results indicate the degradation of OVA to smaller fragments including cleavage of the peptide bonds. In order to observe the degraded OVA molecules clearly and confirm cleavage of the OVA molecule by high-temperature heating, SDS-PAGE with a Tris-Tricine buffer system was performed.

The band with lower molecular size than that of OVA is not clearly apparent in the native PAGE results shown in Figure 2, while smaller fragments were observed by SDS-PAGE. As the cleavage of the molecules by heating was only nicking and the shape of the molecule was maintained after nicking, native PAGE possibly gave no bands for the smaller fragments.

Figure 4 shows that the unheated and heated OVA protein solutions up to 128°C gave only a sharp clear band of the monomer. High mobility of the bands was clearly found in the samples heated to 140–180°C. The protein solutions heated to 140°C and 150°C...
Fig. 3. SDS-PAGE with (left) and without 2-ME (right) for the Unheated OVA Sample and the OVA Heated to Different Temperatures as defined in Fig. 2.

Fig. 4. SDS-PAGE in Tris-Tricine Buffer System of the Unheated and Heated OVA to Different Temperatures as defined in Fig. 2.

Fig. 5. Apparent Viscosity of the Unheated and Heated OVA Samples at pH 7.5 and 10 mg/ml.

provided some pale bands below the monomeric band. Heating to 160°–180°C increased the intensity of these bands with the same pattern as that from heating to 140°–150°C, but additional high-mobility bands increased together with the faint smear. These results indicate cleavage of the OVA molecule, due to hydrolysis of the peptide bonds, at preferential loci in the OVA molecule.

The results of native-PAGE and SDS-PAGE indicate that the thermal denaturation shown as the first endothermic transition peak by DSC measurements induced aggregation of the OVA molecules, before the polymers were formed through intermolecular disulfide linking. The molecular aggregates and the OVA molecules decomposed at a temperature above 140°C. These molecular events must have corresponded to the second endothermic peak by DSC. All the heated protein samples were transparent during heating and cooling at pH 7.5. The formation of molecular aggregates and the degradation should be reflected by the flow behavior of the sample.

Viscosity

The apparent viscosity of each sample heated to various temperatures was measured at shear rate of 76.6, 191.5 and 383.0 s⁻¹ (Fig. 5). The viscosity of the unheated OVA sample and OVA heated to 60°C was similar, while the viscosity of the OVA sample heated to 88° and 93°C was significantly higher. This distinguishable change in the viscosity of the sample heated to 88°C was caused by thermal denaturation, aggregation, and polymerization of the OVA molecules. The viscosity of the samples heated to 113°–140°C was similar to that of the sample heated to 88°C, although some decrease in viscosity was observed at 113°C. This means that heating to around 130°C may induce a change in size of the aggregates, which would influence the viscosity. The viscosity was further reduced in the samples heated to 150°C.
–160°C and sharply decreased in the sample heated to 180°C. This confirms that the OVA aggregates and molecules became dissociated and degraded by heating to above 140°C, as indicated by the electrophoresis results.

Discussion

The heated OVA samples were transparent during heating up to 180°C and cooling to room temperature at pH 7.5. It is known that OVA heated to below 100°C gave a soluble aggregate at acid, neutral and basic pHs, which are separate from the isoelectric point, e.g. pH 7.5, resulting in a transparent aqueous liquid. This is interpreted as a molecular interaction among OVA molecules being suppressed to not form a large insoluble coagulum but to form a soluble linear aggregate under salt-free and the given pH conditions. The OVA samples heated above 100°C in this study also maintained its transparency at pH 7.5. Similar results have been observed with β-lactoglobulin, which also maintained a transparent aqueous liquid state when heated above 100°C at pH 7.5. However, the transparency depended on the pH value; that is, when the pH value of the β-lactoglobulin sample was adjusted to 6.4, the heated sample became a turbid suspension at 93°C. The β-lactoglobulin samples were also turbid at any temperature above 100°C. The size and shape of the aggregates depended on the temperature, changing at around 120°C.

The OVA solution was heated to 110°C (Fig. 1-ii) and, after cooling, was heated again to 180°C. The endothermic peak appeared at around 140°C, which was slightly broader and higher than that in Fig. 1-i (Fig. 1-iii). This might have been due to the effect of cooling the sample in the experiment depicted in Fig. 1-iii, which may have induced a change in the conformational change during cooling that gave a different conformation from that in Fig. 1-i. Therefore, the shape and temperature of the peak may also have been altered.

A high-temperature treatment above 90°C denatured the protein, by which some hydrophobic and other amino acid residues buried inside of the OVA molecule would be exposed to the surface of the OVA molecule, and thus interaction of these amino acid residues by the hydrophobic effect would induce the molecular aggregation. It is not known whether such an effect occurs and how it affects molecular interaction among the denatured molecules at temperatures higher than 100°C. In addition, high temperature would induce the deamidation of amino acid residues, and hydrolysis of the peptide bond would occur, as shown in this study. It has been reported that the rate of a hydrolysis reaction depends upon the local amino acid sequences and higher-order structure of a protein. These reactions proceed slowly or not at all when the protein conformation is intact, especially in the neutral pH region. In this study, hydrolysis of the peptide backbone occurred and the sites of some particular amino acid residues became preferentially susceptible to hydrolysis at around 140°C, because several specific bands with lower molecular size than that of the monomer of OVA were observed in the sample heated to around 140°C, as shown by SDS-PAGE (Fig. 5). When heated to a high temperature in the range of 120°–150°C, the protein molecules would adopt a random-like structure; therefore, specific cleavage loci within the polypeptide chain are presumed to depend on the properties of each residue.

The most susceptible cleavage site of the peptide bond by heating might be at the Asp residues through succinimide formation in protein and peptides. The other labile amino acid residues, which can also undergo cleavage, are Asn, Glu and Gln. Since this reaction has been observed in some cases, such reaction should be applicable to OVA. Although the cleavage site has not been identified, OVA might have been cleaved at the Asp residue site in the molecule as OVA contains 30 residues of Asp and Asn in the molecule. The serine residue has also been recently reported as another susceptible residue, although the mechanism has not been clarified.

Identification of cleavage sites at high temperature may provide comprehensive information on protein denaturation and degradation. The development of a procedure to follow the molecular changes and functionality during heating above 100°C will be important. Hence, clarification of the mechanism for change in the protein properties at high temperature and an analysis of the molecular interaction among protein molecules at high temperature in an enclosed moist system will have critical significance for food processing in order to produce high-quality foods.

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