JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Role of Amyloid Type Cross β-Structure in the Formation of Soluble Aggregate and Gel in Heat-Induced Ovalbumin

H. AZAKAMI, A. MUKAI, AND A. KATO*

Department of Biological Chemistry, Yamaguchi University, Yamaguchi 753-8515, Japan

The heat-induced denaturation curve of ovalbumin followed by the ellipticity at 222 nm in circular dichroism spectra was consistent with that monitored by fluorescence with thioflavin T, which is an indication of amyloid fibril formation, while other proteins such as lysozyme and ovotransferrin did not fluoresce with thioflavin T during heat denaturation. The amount of soluble aggregate formed during heat denaturation was proportional to the increase in fluorescence with thioflavin T. The binding of soluble aggregates with thioflavin T was greatly suppressed in heat-denatured ovalbumin in the presence of thioflavin T. The similar inhibition effect of thioflavin T on the gel formation of heat-induced ovalbumin was observed. These results suggest that the amyloidogenic intermolecular β-structure is involved in the formation of soluble aggregate and gel of heat-induced ovalbumin.

KEYWORDS: Ovalbumin; soluble heat-denatured aggregate; heat-induced gel; amyloid fibril formation; thioflavin T

INTRODUCTION

Because ovalbumin forms soluble aggregates when its aqueous solution at neutral pH is heated, ovalbumin has been studied as a model of irreversible denaturation (*1*−*4*). It has been reported that the soluble ovalbumin aggregates are formed predominantly by hydrophobic interaction, and a kind of regular structure is formed during the progress of the aggregation (*1*, *3*). Painter and Koenig (*5*) first observed the formation of intermolecular cross β-structure during thermal denaturation of various egg white proteins by Raman spectroscopy. Clark et al. (*6*) also reported a similar observation by the infrared and laser Raman spectroscopic study during the heat-induced gelation of a number of globular proteins. We (*3*) have also observed from the simulation of a circular dichroism (CD) curve-fitting program that the formation of a β-sheet structure of heat-denatured ovalbumin increases in a higher protein concentration and salt concentration in which the intermolecular interaction is enhanced.

Recently, protein aggregation is a very hot topic in medical science, because an increasing number of disorders, including Alzheimer's and Parkinson's diseases, the spongiform encephalophathies, and type II diabetes, are directly associated with the deposition of such aggregates in tissues (*7*). It has been reported that the soluble forms of the 20 or so proteins are involved in this well-defined amyloidosis. The aggregate forms of amyloidogenic proteins have many characteristics in common. Amyloidosis shows specific optical behaviors on binding certain dye molecules such as Congo red and thioflavin T (*8*, *9*). The fibrillar structures typical of many amyloidogenic proteins have similar morphologies and a characteristic cross β-sheet whose strands run perpendicular to the fibril axis. The ability of proteins to form an amyloid structure is not restricted to specific amyloidogenic proteins (*10*), and it seems to be a generic feature of polypeptide chains. Koseki et al. (*4*) reported that large aggregated polymers were formed in heat-induced ovalbumin and long linear polymers without branches were observed by transmission electron microscopy. The observation suggests that proteins form in common amyloid type cross β-structures by heat denaturation given an appropriate condition. This paper describes the possibility of amyloid fibril formation in heat-induced ovalbumin.

MATERIALS AND METHODS

Preparation of Ovalbumin and Heat-Denatured Ovalbumin. Ovalbumin was crystallized with sodium sulfate from fresh egg white and recrystallized five times. The purity was confirmed to be more than 99% by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Thermal denaturation of ovalbumin was carried out in 67 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.05% sodium azide. Soluble heat-induced ovalbumin aggregates were prepared as follows. A 5 mL amount of 0.1% ovalbumin solution was put in a test tube with an internal diameter of 15 mm and immersed in a water bath. The ovalbumin solution was heated to a given temperature at the rate of 3 °C/min, the test tube was put into an ice−water bath, and high-performance liquid chromatography (HPLC) and fluorescence measurements were carried out at room temperature near 25 °C. Lysozyme and ovotranferin were used as control proteins. Lysozyme was crystallized from fresh egg white at pH 9.5 in the presence of 5% NaCl and recrystallized several times until no contaminant proteins were observed. Ovotransferrin was obtained from Sigma Co. The purity of ovotransferrin was more than 98%.

CD Measurement of Heat-Denatured Ovalbumin. After filtration through a membrane filter (0.45 μm), 0.1% ovalbumin solution was used for CD measurement. CD measurements were carried out with a

* To whom correspondence should be addressed. Tel: +81 839 33 5852. Fax: +81 839 33 5820. E-mail: akiokato@yamaguchi-u.ac.jp.

10.1021/jf049325f CCC: $30.25
Published on Web 01/27/2005

Jasco J-500 recording spectropolarimeter equipped with a data processor (model DP-501) using a cell with a 0.09 mm light path in the wavelength of 222 nm. The denaturation curve was automatically drawn from 60 to 85 °C. The values of ellipticity obtained from CD spectra were expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$).

Fluorescence Measurement of Heat-Denatured Ovalbumin with Thioflavin T. Fluorescence measurements were carried out using a Hitachi 650-10S Fluorescence Spectrophotometer in a temperature-controlled cell holder. Thioflavin T binding assays were performed by using a modification of the method of Levine (*11*). The fluorescence of thioflavin T was excited at 440 nm with a slit width of 2.5 nm, and the emission was measured at 482 nm with a slit width of 5 nm. To a 0.2 mL amount of heat-denatured ovalbumin solution was added 1.8 mL of thioflavin T (65 μM) solution freshly prepared through a 0.2 μm filter before use to remove insoluble particles.

Measurement of Heat-Denatured Ovalbumin Soluble Aggregate. The amount of soluble aggregate in heat-denatured ovalbumin at a given temperature was determined by HPLC using a Hitachi L-6000 on a TSK gel G3000SW column (Toso, 0.75 cm × 30 cm). The peak area of the higher molecular size peak that appeared in the heated ovalbumin was measured as a soluble aggregate.

Measurement of the Gel Strength of Heat-Denatured Ovalbumin. A 7.5% ovalbumin solution was prepared to determine the gel strength of heat-denatured ovalbumin in 20 mM phosphate buffer (pH 7.0). Ovalbumin solution (1.5 mL) was put into glass tubes (6.0 mm in diameter) previously treated with Sigmacote (Sigma Chemical Co.). The contents of each tube were degassed by placing in a Sharp sonicater (model UT-205, Tokyo) under vacuum for 20 min. The tubes were sealed and heated for 10 min in a water bath at 80 °C. The tubes were taken from the hot bath and cooled at room temperature. The gel was removed from each tube without disrupting the gel surface. Each gel was cut into uniformly flat 5.0 mm thick sections and measured by a tensile tester (Tension UTM-U, Tokyo Baldwin Co., Tokyo) as previously described (*12*). The gel strength was expressed as breaking stress (g).

RESULTS AND DISCUSSION

Ovalbumin forms soluble aggregates by heating at 85 °C in 10 mM phosphate buffer, pH 7.4 (*1*). The turbidity of heat-denatured ovalbumin was not observed by the absorbance at 500 nm, although those of heat-denatured lysozyme and ovotransferrin were slightly observed during heating. Therefore, the structural changes can be followed using CD and fluorometric analysis during heat denaturation without the turbidity of protein solution. Recent studies on the amyloidogenic proteins have demonstrated that the cross β-structure specific for amyloid formation can be monitored by the fluorescence (*8*, *9*) of thioflavin T. Thus, the formation of cross β-structure was monitored by the fluorometric measurement using thioflavin T during heat denaturation of ovalbumin, lysozyme, and ovotransferrin. As shown in **Figure 1**, the fluorescence of thioflavin T for ovalbumin greatly increased during heat denaturation, while that for lysozyme and ovotransferrin did not increase.

This observation suggests that ovalbumin forms the cross β-structure during heat denaturation. Correlation of the formation of cross β-structure with the unfolding was investigated in heat-denatured ovalbumin. **Figure 2** shows the denaturation curve of heat-induced ovalbumin followed by the ellipticity at 222 nm in CD spectra and the fluorescence intensity with thioflavin T. The former is an indication of the structural changes in denaturation, and the latter is an indication of the amyloid fibril formation specific to the cross β-structure. As shown in **Figure 2**, the denaturation curve of heat-induced ovalbumin followed by the ellipticity at 222 nm in CD spectra was consistent with that monitored by fluorescence with thioflavin T that is an indication of amyloid fibril formation. This result suggests that the cross β-structure is formed as the progress of unfolding in

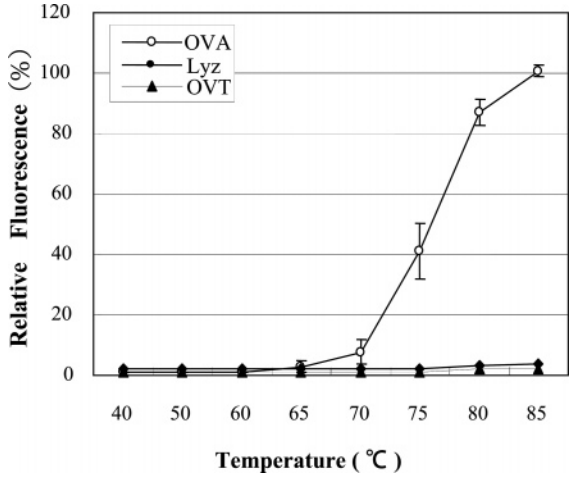

Figure 1. Changes in the fluorescence of thioflavin T for ovalbumin (○), lysozyme (●), and ovotransferrin (◆) during heat denaturation. Error bars indicate the standard deviations ($n = 5$).

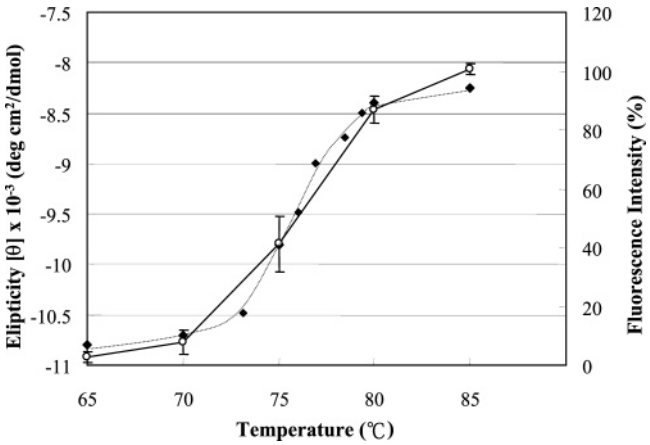

Figure 2. Relationship between ellipticity at 222 nm (◆—◆) and fluorescence intensity with thioflavin T (○—○) during heat denaturation of ovalbumin. Ovalbumin solution (0.2 mL) heated at a given temperature was diluted into 1.8 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 65 μM thioflavin T, and then, the fluorescence intensity was measured at 482 nm with exitation at 440 nm. The ellipticity was obtained from CD spectra at 222 nm. Error bars indicate the standard deviations ($n = 5$).

heat-denatured ovalbumin. These results suggest that only ovalbumin specifically forms amyloid type cross β-structure during heat denaturation. To further elucidate the mechanism, the relationship between the formation of soluble aggregate and the cross β-structure during heat denaturation was investigated. **Figure 3** shows the HPLC patterns of heat-denatured ovalbumin. The peak of unheated ovalbumin eluted at an elution time of 29 min, while the peak of heat-denatured soluble aggregates eluted at an elution time of 17 min, corresponding to the void volume of a column of TSK Gel G3000SW. **Figure 4** showed the relationship between the peak area of soluble aggregates and the fluorescence intensity with thioflavin T for heat-denatured ovalbumins. As shown in **Figure 4**, the amount of soluble aggregate formed during heat denaturation was proportional to the increase in fluorescence with thioflavin T, suggesting that the formation of soluble aggregates was closely involved in the formation of the amyloid type cross β-structure. It has been reported in the research on the amyloidogenic protein that thioflavin T specifically interacts with amyloid β-sheet structures. This suggests that the formation of ovalbumin soluble

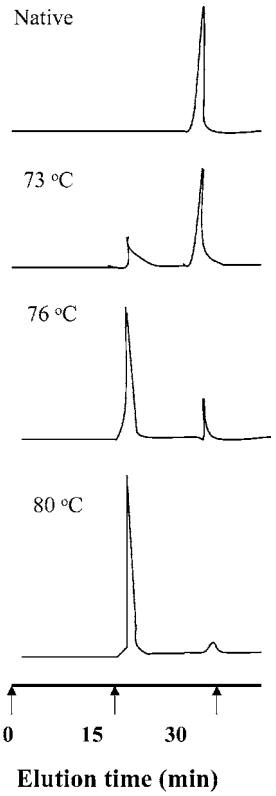

Figure 3. HPLC patterns of ovalbumin during heat denaturation. A 0.1% ovalbumin solution was heated in a 67 mM phosphate buffer, pH 7, containing 0.1 M NaCl.

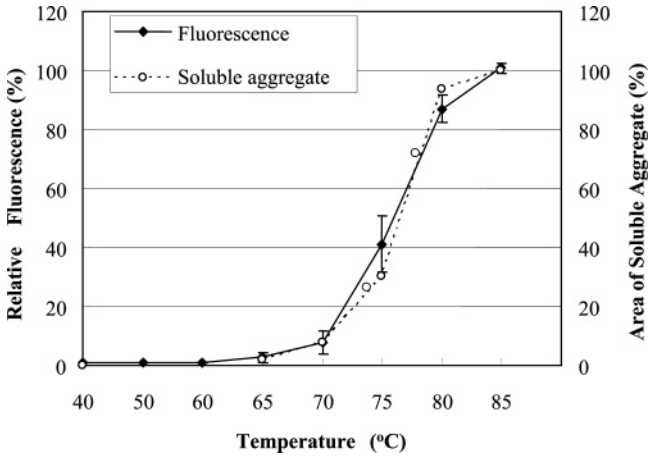

Figure 4. Relationship between fluorescence intensity with thioflavin T and area of soluble aggregate during heat denaturation of ovalbumin. The area of soluble aggregates was calculated from HPLC patterns. Error bars indicate the standard deviations ($n = 5$).

aggregate might be affected in the presence of thioflavin T during heat denaturation. The coexistent thioflavin T binds to the β-structure exposed on the molecular surface during heat denaturation, thereby suppressing the subsequent formation of the cross β-structure. Therefore, it is expected that thioflavin T affects the formation of soluble aggregate and gel of heat-denatured ovalbumin. As expected, the denaturation curves of ovalbumin followed by the fluorescence intensity with thioflavin T were greatly affected in a concentration-dependent manner of thioflavin T, as shown in **Figure 5**. It is probable that the coexistent thioflavin T binds to the cross β-structure formed during heat denaturation, thereby suppressing the formation of a further extended cross β-structure. However, the considerable

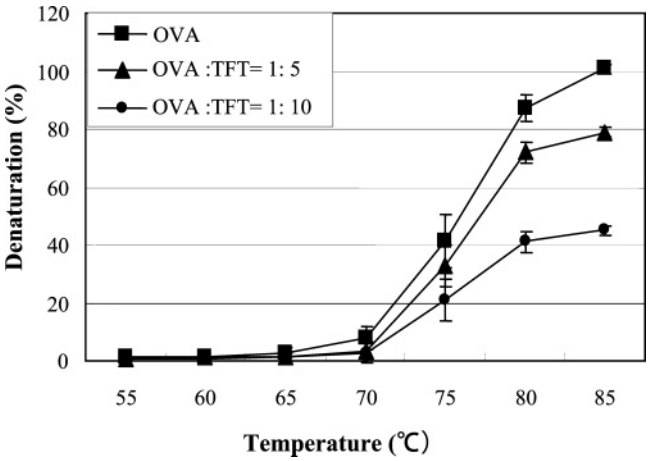

Figure 5. Denaturation curves of OVA in the presence of thioflavin T followed by the measurement of fluorescence of thioflavin T. Ovalbumin solution (0.2 mL) heated at a given temperature was diluted into 1.8 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 65 μM thioflavin T, and then, the fluorescence intensity was measured at 482 nm with exitation at 440 nm. Error bars indicate the standard deviations ($n = 3$).

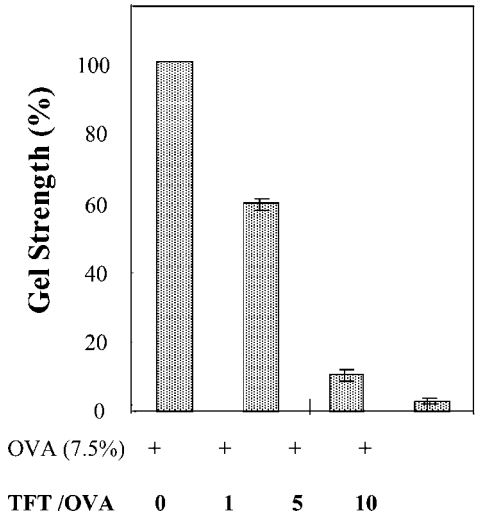

Figure 6. Gel strength of heat-induced ovalbumin in the presence of thioflavin T. The gel of heat-induced ovalbumin was formed by heating 7.5% ovalbumin solution at 80 °C for 10 min. TFT/OVA indicated the molar ratio of thioflavin T to ovalbumin. Error bars indicate the standard deviations ($n = 3$).

amount of soluble aggregate may be formed in the presence of thioflavin T, because the cross β-structure was partially formed in the presence of thioflavin T, as shown in **Figure 5**. It seems likely that the heat-induced aggregates of ovalbumin can be formed by other binding forces such as hydrophobic and electrostatic forces despite the suppression of the cross β-structure. **Figure 6** shows the effect of thioflavin T on the gel formation of heat-induced ovalbumin. The gel strength was greatly decreased in a concentration-dependent manner of thioflavin T. This result indicates that the cross β-structure is essential to the gel formation of heat-induced ovalbumin. It is well-known that the heat-induced gelation of proteins is affected by the balance of various factors such as the exposed hydrophobic surface, electrostatic force, SS bond, etc. The results obtained in this paper strongly support that the amyloid type intermolecular β-structure is closely involved in the formation of soluble aggregate and gel of heat-induced ovalbumin.

In globular proteins, the polypeptide main chain and hydrophobic side chains are largely buried within the folded structure. When proteins are unfolded by heat denaturation, many proteins may possibly convert into amyloid fibril or gel by cross β-structure through hydrogen bonds involving the polypeptide main chains in an appropriate condition. This paper describes the evidence and importance of the cross β-structure in the gel formation of heat-induced ovalbumin, occurring in a similar manner as amyloid fibril formation. The common characteristics of amyloid formation of amyloidogenic proteins are specific optical behaviors on binding certain dye molecules such as Congo red and thioflavin T, and the fibrillar structures typical of amyloid formation have very similar morphologies that are long unbranches and approximately 10 nm in diameter. Doi and Kitabatake (*13*) reported that the diameter of heat-induced ovalbumin linear aggregate is approximately 12 nm. Thus, the optical behavior on binding thioflavin T and morphological behavior on the diameter of ovalbumin soluble aggregate suggests that heat-induced ovalbumin gel is formed through a cross β-structure in a similar manner as amyloid fibril formation.

LITERATURE CITED

(1) Kato, A.; Tsutsui, N.; Matsudomi, N.; Kobayashi, K.; Nakai, S. Effects of partial denaturation on surface properties of ovalbumin and lysozyme. *Agric. Biol. Chem.* **1981**, *45*, 2755−2760.

(2) Egelandsdal, B. Conformation and structure of mildly heat-treated ovalbumin in dilute solutions and gel formation at higher protein concentrations. *Int. J. Pept. Protein Res.* **1986**, *28*, 560−568.

(3) Kato, A.; Takagi, T. Formation of intermolecular β-sheet structure during heat denaturation of ovalbumin. *J. Agric. Food Chem.* **1988**, *36*, 1156−1159.

(4) Koseki, T.; Kitabatake, N.; Doi, E. Irreversible thermal denaturation and formation of linear aggregates of ovalbumin. *Food Hydrocolloids* **1989**, *3*, 123−134.

(5) Painter, P. C.; Koenig, J. L. Raman spectroscopic study of the proteins of egg white. *Biopolymer* **1976**, *15*, 2155−2166.

(6) Clark, A. H.; Saaunderson, D. H.; Suggett, A. Conformation and structure of mildly heat-treated ovalbumin in dilute solutions and gel formation at higher protein concentrations. *Int. J. Pept. Protein Res.* **1981**, *17*, 353−364.

(7) Dobson, C. M. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* **1999**, *24*, 329−332.

(8) Morozova-Roche, L. A.; Zurdo, J.; Spencer, A.; Noppe, W.; Receveur, V.; Archer, D. B.; Joniau, M.; Dobson, C. M. Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants. *J. Struct. Biol.* **2000**, *130*, 339−351.

(9) Arora, A.; Ha, C.; Park, C. B. Inhibition of insulin amyloid formation by small stress molecules. *FEBS Lett.* **2004**, 121−125.

(10) Dobson, C. M. Protein folding and misfolding. *Nature* **2003**, *426*, 884−890.

(11) Levine, H. Thioflavin T interaction with amyloid β-sheet structures. *Amyloid* **1995**, *2*, 1−6.

(12) Kato, A.; Ibrahim, H. R.; Watanabe, H.; Honma, K.; Kobayashi, K. New approach to improve the gelling and surface functional properties of dried egg white by heating in dry state. *J. Agric. Food Chem.* **1989**, *37*, 433−437.

(13) Doi, E.; Kitabatake, N. Preparation of transparent gels from egg white and egg proteins. In *Egg Uses and Processing Technologies*; Sim, J. S., Nakai, S., Eds; Cab International, 1994; pp 269−282.

Received for review April 27, 2004. Revised manuscript received November 27, 2004. Accepted December 7, 2004.

JF049325F