Abstract: β-Lactoglobulin A solution at pH 6.4 was heated to 180 °C at the rate of 6 °C/min. By differential scanning calorimetry two independent endothermic peaks were observed. The first peak appeared below 100 °C is corresponding to the thermal denaturation of protein. This conformational change led to the aggregation and polymerization of molecules through disulfide linkage, particularly observed above 100 °C. The second endothermic peak appeared around 150 °C, which was brought by the decomposition of molecules as judged from electrophoresis. Up to 100 °C the viscosity of β-lactoglobulin A solution increased by heating, while the viscosity was reduced beyond 113 °C, due to change in the size of aggregate and decomposition of β-lactoglobulin A molecules.

Key Words: β-Lactoglobulin A, Heating at high temperature, heat denaturation, heat aggregation.

Introduction

Heating of foods at high temperature above 100 °C is a general method of food processing used not only in such traditional techniques as roasting, baking, and frying, but also in modern food processing as retort cooking, sterilization, and extrusion cooking (Finley 1989; Boye and others 1997a). In the traditional cooking methods, heating is performed in an open system, that is, at atmospheric pressure. Most foods contain water, and by its evaporation through heating the food temperature is maintained at 100 °C. Without water, food components oxidize and burn. On the other hand, heating foods that contain water in a closed system makes it possible to keep water molecules in the system, resulting in temperatures above 100 °C, high pressure, and moist conditions. In particular, high-temperature short-time heating of foods at high pressure in a closed system is a typical method of modern-day food processing. This versatile and sophisticated processing is popular in food mass production in the canned and process foods industries, including milk processing (Finley 1989; Nakai and Li-Chan 1989; Boye and others 1997b).

Heating is performed to sterilize foods by killing bacteria, but at the same time other food components are affected. Chemical and physicochemical studies on the effects of high-temperature heating on foods and food components have been done from various viewpoints in order to determine the most desirable heating conditions. However, the effects of heating at higher temperatures than 100 °C on food components are not well known, compared to the effects of heating below 100 °C (Daniel and others 1996).

β-Lactoglobulin (β-LG) is the major milk whey protein component and is used as a food ingredient for various kinds of processed and/or fabricated foods. Extensive research has been carried out on the heating of β-LG, and molecular analysis of the thermal denaturation of β-LG has been reported in the field of food science as well as that of protein chemistry, in which β-LG is a noted model protein (Iametti and others 1995; Hoffmann and others 1997; Morgan and others 1999). High-temperature heating of protein generally results in degradation and burning when it is done in an open system, whereas in closed system with water, e.g., in extrusion cooking, water molecules serve as a plasticizer in protein materials, and viscosity increases at higher temperature.

It has been reported that some endothermic reactions occur at around 140 °C in β-LG, but not accompanied by any change in amino acid composition of the β-LG molecule (De Wit and Swinkels 1980; De Wit and Klarenbeek 1981). These authors conjectured that heating β-LG above 100 °C accelerated the polymerization of β-LG molecules through disulfide linkage, and that heating at higher temperatures would break down this linkage endothermically.

In our study we aimed to characterize the molecular changes in purified β-lactoglobulin A (β-LG A) by heating at temperatures above 100 °C, as well as to learn about the relation of these changes to the flow behavior of β-LG A, thereby contributing to our fundamental knowledge of high-temperature treatment in modern food processing.

Materials and Methods

Preparation and heating of β-lactoglobulin A

β-LG was purified from whey protein concentrate using a previously described method (Kinekawa and Kitabatake 1996), and β-LG A was obtained as follows. Purified β-LG (6,200 to 6,500 mg) was applied on DEAE (DEAE Cellulofine A-500, Seikagaku Co., Tokyo, Japan) anion exchange column (30 × 360 mm) chromatography to separate the β-LG variant (β-LG A) from other components. Elution was performed with 1 L linear gradient from 100 mM to 250 mM Tris-Cl buffer, containing 0.1 mM EDTA-2Na (pH 7.2) at a flow rate of 2.0 to 2.2 mL/min and 4 °C. Fifteen-mL fractions were collected and the purity of β-LG A in each fraction was determined with polyacrylamide gel electrophoresis without denaturant (native-PAGE). The fractions giving the single band of β-LG A by native-PAGE were collected and combined. The β-LG A obtained was exhaustively dialyzed against distilled, deionized water. After dialysis, the pH of β-LG A solution was adjusted to 7.5 by addition of diluted NaOH before drying in a centrifugal vaporizer (Eyela CVE-100D; Tokyo Rika Co., Tokyo, Japan) under reduced pressure. The lyophilized powder was stored at −20 °C until use. The powder was dissolved in distilled, deionized water to a final concentration of 10 mg/mL. The final pH of the β-LG A so-
lution was 6.4. The β-LG A solution (1.2 mL) was transferred into glass vials (length 3.2 cm; diameter 1.0 cm). They were tightly closed and heated in a temperature-regulated oil heater at the rate of 6 °C/min. The temperature of the sample in each glass vial was corrected by the temperature measured with a thermo-label indicator (Nishiyu Gigen Kogyo Co., Ltd., Tokyo, Japan) placed inside of the vial. The exact temperature of the sample in the vial can be indicated by the color of the label changed at a given temperature. Samples were heated to 180 °C. All Chemicals used were guaranteed reagent grade.

Differential scanning calorimetry (DSC)
Thermal analysis of β-LG A was performed by differential scanning calorimetry (DSC) using a DSC 100 calorimeter (Seiko Instruments Inc., Chiba, Japan). Silver pans (No. 560-003; Seiko Instruments, Tokyo, Japan) were heated before use at 170 °C for 1 h in a special stainless steel autoclave chamber saturated with water vapor. All trials were performed as follows except where noted. The β-LG A powder was dissolved in distilled, deionized water to a final concentration of 60 mg/mL and final pH of 6.4, and then 50 μL of the solution was placed in a silver pan, and the pan was sealed tightly with a fitted lid. Fifty mL of distilled, deionized water was used as a reference. Heating was carried out at a rate of 6 °C/min from 25°C to 180 °C. The data sampling time interval was 0.5 s. Indium was used to calibrate the instrument. The data were stored in a diskette and analyzed with a DSC analysis software. The onset temperature of heat absorption, the peak of heat absorption, and the heat of transition or enthalpy of denaturation (ΔH) were computed from the thermogram.

Viscosity measurement
The viscosity of the heated sample was measured using a cone-plate type rotational viscometer (Model E viscometer, Tokyo Keiki Co. Ltd., Tokyo, Japan). The cone jacket of the viscometer was maintained at 25 ± 0.2 °C by circulating of water from a constant temperature bath. The cone had a radius of 24 mm and a cone angle of 1°34’’. The viscosity was measured immediately after the samples were cooled to room temperature. One mL of the sample was poured into the cone vessel of the viscometer, and the solution was incubated for 3 min before rotation. Shear rate was changed from 9.6 s⁻¹ to 76.6 s⁻¹. In this study we used an apparent viscosity at the shear rate of 9.6 s⁻¹ and 38.3 s⁻¹.

Gel electrophoresis
Native-PAGE was carried out according to Davis (1964) on 10% acrylamide separating gel and 4.5% acrylamide stacking gel. The β-LG A sample solution was mixed with glycerol. The protein and glyc erol concentrations were adjusted to 0.125 mg/mL and 20% (v/v), respectively. Exactly 2.5 μg of protein was loaded onto each lane. The gels were stained with 0.1% (w/v) Coomassie brilliant blue R 250 in 50% methanol. Polyacrylamide gel electrophoresis with denaturant (SDS-PAGE) was conducted according to the method of Laemmli (1970) on 13.5% acrylamide separating gel and 3.0% acrylamide stacking gel. The protein sample with and without 2-mercaptoethanol (2-ME) were heated at 100 °C for 5 min to complete denaturation and then cooled to room temperature.

Particle size analysis
The particle size distribution of heated β-LG A was measured using a laser diffraction particle-size distribution analyzer (Model LA-500, Horiba Ltd., Kyoto, Japan). Multiple scattering effects were avoided by dilution of the heated samples to 0.6 to 0.8 mg protein/mL. Dilution was carried out using distilled, deionized water. Seven mL of diluted sample was put into a cuvette cell for measurement. This instrument measures the angular dependence of the intensity of light scattered from a dilute heated β-LG A sample and then determines the particle size distribution that gives the closest fit between theoretical calculations (Mie theory) and experimental measurements.

Amino acid analysis
Amino acid composition of the sample was analyzed by an amino acid analyzer (Model L-8500A High Speed, Hitachi Ltd., Tokyo, Japan). The β-LG A sample (25 μg) was hydrolyzed with 6 N HCl containing 1% (v/v) phenol for 22 h under reduced pressure at 110 °C. After hydrolysis, HCl was completely evaporated and the residue was dissolved in sodium citrate buffer, pH 2.2. An amount of sample equivalent to 12.5 μg (0.68 nmol) of β-LG A was analyzed. The amino acids were detected with postcolumn derivatization with ninhydrin.

Pepsin treatment
β-LG A samples were adjusted to pH 2 by 1 N HCl and diluted to a final concentration of 0.26 mg/mL. They were then pre-incubated at 37 °C for 10 min. Porcine pepsin (activity: 2,500 to 3,100 U/mg of protein; P-7012) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The lyophilized pepsin was dissolved in distilled, deionized water then added to pre-incubated protein solution with a protein to enzyme ratio of 100:1 (wt/wt). The enzyme reaction was carried out at 37 °C for 1 h and stopped the reaction by addition of SDS buffer (pH 7.3) and boiling. SDS-PAGE with and without 2-ME was used for analysis of the products of enzyme reaction.

Results and Discussion

Differential scanning calorimetry (DSC)
DSC was used to examine the effects of heating on β-LG A dissolved in distilled, deionized water. The thermogram of β-LG A solution (60 mg/mL, pH 6.4) heated from 25 °C to 180 °C at a rate of 6 °C/min gave thermal transition peaks at around 90 °C and 150 °C (Figure 1-i). To clarify whether these two peaks were related to each other, a new sample of β-LG A was first heated from 25 °C to 110 °C at a rate of 6 °C/min (Figure 1-ii) and cooled to 25 °C at a rate of about 0.8 °C/min, and then heated again to 180 °C at a rate of 6 °C/min (Figure 1-iii). The thermogram shows that β-LG A developed the peak at around 80 °C (Figure 1-ii) and by the second heating no peak was observed at around 80 °C, while another peak appeared at around 150 °C (Figure 1-iii). After cooling, the sample was heated again to 180 °C (Figure 1-iv). This third heating gave no peak up to 180 °C (Figure 1-iv). Judging from these results, the two endothermic peaks were independent, representing two different kinds of reaction that occur in protein molecules when heating to 180 °C; and these reactions are apparently irreversible over the time frame of the experiment. The first endothermic peak must correspond to the thermal denaturation of β-LG A (De Wit and Swinkels 1980; De Wit and Klarenbeek 1981; Park and Lund 1984; Qi and others 1995). To clarify the molecular event in the second reaction as well as the first endothermic reaction, β-LG A samples heated at various temperatures were analyzed by native-PAGE and SDS-PAGE.
Analysis by polyacrylamide gel electrophoresis (native-PAGE)

β-LG A solution (10 mg/mL) was placed in a glass vial and heated to one of several temperatures at the same rate as used for DSC, and then analyzed by native PAGE (Figure 2). The β-LG A sample heated to 88°C gave several bands on separating gel (between band B and β-LG A) with lower mobility than that of β-LG A monomer. The intensity and number of bands with lower mobility than that of β-LG A increased with an increase in temperature. Bands could also be observed on the top of stacking gel (band A). The sample heated to 113°C gave the intense bands with low mobility, suggesting the formation of aggregates of β-LG A. A pale band was observed between the stacking and separating gel (band B), indicating some β-LG A makes huge aggregates. At temperatures above 113°C, the intensity of such bands in separating gel weakened, and at 180°C the bands grew faint, suggesting that β-LG A was decomposed by heating at these temperatures. We next clarified the details of change in β-LG A molecule and the intermolecular linkage by SDS-PAGE with and without 2-ME.

SDS-PAGE

SDS-PAGE with 2-ME (Figure 3-left) revealed a single and sharp band of β-LG A monomer in the unheated and heated samples at 40, 60, and 88°C. The β-LG A sample heated to 93°C, 113°C, 128°C, and 140°C also gave a band corresponding to β-LG A monomer with higher density than those of the samples heated at 150°C and 160°C. Heating at 180°C gave no band of protein stained by Coomassie brilliant blue R 250 (Figure 3) and silver staining (data not shown) in the separating gel. A pale band was observed between the stacking and separating gel (band b in Figure 3) in the range from 113°C to 180°C, suggesting that the large polymer of β-LG A was formed through non-disulfide covalent intermolecular linkage. Such a band was not observed on heating at 40°C to 93°C. Furthermore, stained bands were seen on the stacking gel (band a) in the samples heated at 140°C to 160°C as a result of large aggregates of β-LG A, while several bands in separating gel with higher mobility than β-LG A could be found on the same lanes. This means that the peptides, being smaller than β-LG A, were formed by heating at 160°C to 180°C and indicates that β-LG A and/or polymerized β-LG A were subjected to undergo decomposition as well as dissociation. Heating at 180°C gave a very pale band on the stacking gel (band a in Figure 3) and between stacking gel and separating gel (band b). Furthermore, no band could be observed in separating gel, indicating that β-LG A and its polymer were decomposed to smaller peptide components, which should have diffused out of the separating gel as their molecular size was small enough to pass through the matrix of the gel. To clarify the aggregates found in the heated sample, SDS-PAGE without 2-ME was carried out (Figure 3-right). Bands for polymerized β-LG A were observed at temperatures higher than 88°C (Figure 3-right). These polymers seemed to be formed by disulfide linkage, based on the absence of such bands between band b and the band of β-LG A in SDS-PAGE with 2-ME (Figure 3-left). There is a distinct band on the stacking gel (band a in Figure 3-right) in the case of the samples heated at above 88°C. These huge aggregates seemed to be mostly formed by intermolecular disulfide linkage among β-LG A molecules, the decomposed β-LG A molecules, and/or both. From these results β-LG A was thermally denatured at around 80°C, and aggregation and polymerization occurred through intermolecular disulfide linkage. At higher temperature, that is, around above 140°C, decomposition of β-LG A molecule, including hydrolysis of peptide bond, occurred.

Viscosity

Heating of β-LG A at different temperatures influenced the degree of polymerization, aggregation, and decomposition. The incidence of molecular change by heating could affect functional properties of β-LG A. Viscosity is one of the functional properties of food protein and also sensitive indicator for polymerization and decomposition of protein mol-

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**Figure 1**—DSC thermograms of β-LG A in the temperature range of 25°C to 180°C. The protein was dissolved in distilled, deionized water. The pH of the protein solution was 6.4. i) β-LG A (60 mg/mL) was heated from 25°C to 180°C at a rate of 6°C/min. ii) β-LG A (60 mg/mL) was heated from 25°C to 110°C. iii) The sample of ii) was cooled to 25°C and heated again to 180°C. iv) After cooling the sample of iii) was heated again to 180°C.

**Figure 2**—Native PAGE of unheated β-LG A solution (kept at 25°C) and β-LG A solution heated to different temperatures. β-LG A (10 mg/mL, pH 6.4) was heated from 25°C to 180°C at a rate of 6°C/min.
Accordingly, the flow behavior of β-LG A solution heated at various temperatures was determined. Based on the results of the DSC measurement, heating led to two independent events in molecules, one at around 80 °C and the other at around 150 °C. The apparent viscosities of the samples heated at various temperatures, measured at a shear rate of 9.6 s⁻¹ and 38.3 s⁻¹, were plotted against temperature (Figure 4). The viscosity of the sample slightly increased at 60 °C and 88 °C and steeply increased to 26.6 mPa·s at 93 °C. When the samples were heated at 113 °C to 180 °C, the viscosity gradually decreased. The increase in viscosity up to 100 °C should be due to the polymerization and aggregation of β-LG A as a result of thermal denaturation. The decrease in viscosity above 113 °C appears maybe due to the decomposition of β-LG A. However, it is not clear why viscosity suddenly decreased around and/or above 113 °C, since polymer and aggregates were still observed by gel electrophoresis of the samples treated at and above 113 °C. In order to clarify this matter, the protein particle size of coagulum in the samples was measured.

**Particle size distribution**

Unheated β-LG A solution and the β-LG A solution heated to 88 °C were transparent and devoid of coagulum particle, whereas β-LG A solution heated to above 93 °C became turbid. As shown in Figure 6, the particle distribution of the sample heated to 93 °C contained two distinct peaks corresponding to large (3.90 to 39.23 μm) and small (0.11 to 1.00 μm) coagulum particles. The sample heated to 113 °C also provided 2 peaks, with the peak corresponding to the small coagulum particles and the other peak corresponding to the large particle size, being larger than that for the sample heated to 93 °C, indicating that the size of the coagulum particle by increase from 93 °C to 113 °C. This suggests that the coagulum particles formed on heating above the denaturation temperature became bigger by incorporating the smaller coagulum particles under the increase in temperature. This phenomenon would correspond to the difference in viscosity between 93 °C and 113 °C. At 128 °C and above no peak corresponding to the large coagulum particles was observed; only the peak for smaller particle remained. These results indicate that heating above denaturation temperature; at around 93 °C, induces the polymerization and aggregation of thermally denatured molecules, which in turn causes the increase in viscosity and formation of coagula. At 113 °C the degree of polymerization increased and the coagulum particles became huge, although the latter effect was suddenly decreased at temperature above 113 °C. At higher temperatures β-LG A molecules in aggregates were dissociated and viscosity decreased more.

To further investigate possible differences among these fractions, we also analyzed their susceptibility to pepsin and their amino acid compositions. It is known that native β-LG is resistant to pepsin even at acid pH, while thermally denatured β-LG is easily hydrolyzed by pepsin (Kinekawa and Kitabatake 1998). The β-LG A samples heated at temperatures up to 180 °C was treated by pepsin. The unheated β-LG A was not hydrolyzed by pepsin while the heated β-LG A was easily hydrolyzed. The β-LG A heated at higher temperature was more readily hydrolyzed to smaller molecules (Figure 6).

The results of the amino acid analysis showed no remarkable change in the amino acid composition of β-LG A by heating (data not shown). Therefore, the degradation at high temperatures above 140 °C in the β-LG A molecule seems to hydrolyze peptide bonds, but not induce a striking decomposition of specific amino acid residues.

This study revealed that two remarkable events occur during heating of β-LG A up to 180 °C. De Wit and others (1980, 1981) also mentioned that two endothermic peaks appearing during heating of β-LG A, and concluded that the first endothermic peak appeared at around 80 °C was a result of thermal denaturation of β-LG, while the second endot-
hermic peak at around 140 °C was due to reduction and decomposition of the disulfide bridge formed concomitantly by thermal denaturation. In the present study, however, peptide bonds in β-LG molecules were cleaved at temperatures above 140 °C. Qi and others (1995) suggested the β-LG A molecule changed from native state to the molten-globule state at 70 °C and became to denatured state at around 140 °C. However, the results of this study indicate that the endothermic reaction at around 140 °C consists of decomposition of the protein molecules, including breakage of peptide bonds.

At 93 °C two peaks were observed, and the peak corresponding to the large-size particle shifted to the left-side in Figure 5 by increase in temperature to 113 °C, meaning that the size of particles in the large-size particle fraction at 93 °C became still larger by increase in temperature to 113 °C. On the other hand, the peak corresponding to the small-size particle at 93 °C became small with an increase in temperature from 93 to 113 °C. This means that the particle distribution pattern of the coagulum changed between 93 °C and 113 °C. Above 128 °C the peak corresponding to the small-size particle became large. Above 128 °C the particle distribution pattern was not changed, that is, only one peak corresponding to the small-size particle was observed, and the peak height was not changed.

Heat-denatured protein molecules associate together not only by formation of disulfide bridges, but also by noncovalent bond, as shown here by SDS-PAGE with and without 2-ME. At temperatures above 113 °C, SDS-PAGE continued to show an increase in the amount of the high-molecular species on the top of the gel (Figure 3), while the number of large particles decreased (Figure 5). One possible explanation for this finding is that the larger aggregates may have been formed from the association of the smaller aggregates that formed at low temperatures, which is due to the entropy effects with increase in temperature from 93 °C to 113 °C. The entropy of nonpolar group hydration on protein unfolding is negative. Therefore, heat-denatured protein molecules tend to interact together through the contact of hydrophobic amino acid residues exposed on heating, which reduces the hydration with surrounding water molecules and thereby prevents a decrease in the entropy of the system by introducing an ordered structure of water molecules around the protein molecule. This means that, at low temperature, the nonpolar groups of the protein molecules, exposed from inside to the surface by unfolding on heating, interact with the nonpolar groups of other protein molecules. The entropy of hydration of nonpolar groups effect decreases by an increase in temperature from 93 °C to 113 °C. The entropy effect that drives the formation of large aggregates may decrease in magnitude with increasing temperature. The temperature at which the hydration entropy becomes zero for nonpolar groups is 112 °C (Baldwin 1986; Murphy and others 1990; Murphy and Gill, 1991). According to Privalov and Makhatadze (1993), the temperature at which the hydration en-

![Figure 4](image4.png)

**Figure 4**—Apparent viscosities of unheated β-LG A and β-LG A heated to different temperatures. The shear rate of 9.6 s⁻¹ (○), and 38.3 s⁻¹ (●).

![Figure 5](image5.png)

**Figure 5**—Particle size distribution of the β-LG A heated to 93°C (○), 113°C (●), 128°C (■), 140°C (◆), 150°C (★), 160°C (▲), and 180°C (+). Logarithmic scale is used for x-axis.

![Figure 6](image6.png)

**Figure 6**—Pepsin treatment. β-LG A samples (0.26 mg/mL, pH 2) was incubated with porcine pepsin (0.0026 mg/mL) at 37°C for 1 h, and then applied on SDS-PAGE with 2-ME. Details are shown in the text.
tropy becomes zero is 122 °C for the aliphatic groups and 104 °C for the aromatic group. Above 120 °C, the decrease in entropy through the formation of ordered structure of water molecules around the nonpolar group cannot be expected, and thus the protein-protein interaction resulting from the contact of nonpolar groups would disappear. Similarly, the interaction among protein aggregates would weaken and disappear at above 120 °C if the entropy effect is the major force driving this aggregate-aggregate interaction. The change in the particle size distribution of heated samples that occurred at the temperature range of 93 °C to 113 °C and from 113 °C to 128 °C may be interpreted by this effect. Thus, the aggregates would have continued to interact together to form larger aggregates in the temperature range of 93 °C to 113 °C by the entropy effect, while the large aggregates would have collapsed at temperature higher than around 113 °C since the entropy effect is negligible above this temperature.

If the aggregates obtained by heating above around 120 °C could be different from those obtained below around 120 °C, these aggregates would show different properties and food functionalities. Therefore, the protein samples treated by heating above 110 °C should have different properties, including flow behavior, from those heated below 120 °C.

In this study, the pH of β-LG A solution was 6.4, a pH at which heat-denatured β-LG A tends to coagulate, and this must have been responsible for the large and firm coagula at high temperature. The aggregation pattern of heat-denatured β-LG A strongly depends on pH and ionic strength (Kitabatake and Kinekawa 1996). The aggregations of β-LG A at high temperature and weak alkaline pH are also of interest, and are currently under investigation in our laboratories.

References
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