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Estimation of the Molecular Weight Distribution of Heat-Induced Ovalbumin Aggregates by the Low-Angle Laser Light Scattering Technique Combined with High-Performance Gel Chromatography

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Heat-induced ovalbumin aggregates with a wide range of molecular weight distribution were efficiently fractionated by high-performance gel chromatography using a tandem array of TSK gel G4000SW and TSK gel G6000PW columns. Elution from the columns was monitored by a low-angle laser light scattering photometer and a differential refractometer to obtain the molecular weight distribution of ovalbumin aggregates. The weight-average molecular weight of ovalbumin aggregates increased progressively with the upper limit of temperature of heating followed by immediate cooling, as follows: 2.7×10^6 (degree of polymerization, 60) for 78 °C, 6.0×10^6 for 80 °C, and 9.7×10^6 for 82 °C. The molecular weight distribution curve shifted to the side of higher molecular weight and became flatter with an increase in the heating temperature. The average molecular weight increased with prolonged standing at 25 °C after heat treatment at 80 °C as follows: 9.5×10^6 for 6 h and 11.7×10^6 for 22 h.

Proteins are generally aggregated as a result of heat denaturation. Intermolecular interaction behind this phenomenon is an important factor determining functional properties of food proteins such as gelation and foaming property. Ovalbumin forms water-soluble aggregates when its aqueous solution at neutral pH with concentration near 1 mg/mL is heated at a temperature between 80 and 90 °C. It was expected, therefore, that useful information can be obtained from detailed investigation of the formation of the aggregates. Accurate determination of molecular weight of the aggregates is indispensable in such a study. A recently developed method of molecular weight determination using a low-angle laser light scattering technique combined with high-performance gel chromatography (Takagi, 1985) seems to be suitable for such purpose.

In a previous study (Kato et al., 1983), we monitored the elution of heat-treated ovalbumin from a TSK gel G3000SW column using the above measuring system. The heat-induced aggregates were eluted at the void volume. Only weight-average molecular weight could be estimated for the aggregates. Despite the limitation, it was found that the average molecular weight of ovalbumin aggregates increased progressively with temperature of heat treatment, protein concentration, and salt concentration. The observed molecular weights ranged from several million to 10 times of it.

TSK gel PW type columns have been shown to be efficient for the fractionation of polysaccharides with wide molecular weight distribution such as pullulan and amylose (Takagi and Hizukuri, 1984; Hizukuri and Takagi, 1984). We have, therefore, tried application of the column for the fractionation of the heat-induced ovalbumin aggregates and found that a tandem array of TSK gel G4000 SW and TSK gel G6000PW is quite suitable for the fractionation of the aggregates with respect to their size. Monitoring of molecular weight distribution of the heat-induced ovalbumin aggregates was attempted for the first time as well as described below. The results obtained seem to give insight into the initial phase of heat denaturation of proteins, which was first recognized among various modes of protein denaturation but least understood despite its important relevance especially to food processing.

MATERIALS AND METHODS

Ovalbumin was crystallized with sodium sulfate from fresh egg white and recrystallized five times (Kekwick and

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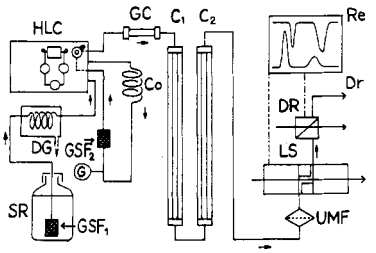

Figure 1. Instrumentation of the measuring system of molecular weight: SR, solvent reservoir (3.0 L); GSF, sintered stainless-steel filter (Umetani Seiki Co., Model SYF); DG, degasser (Elma Optical Works, Model ERC-3310); GSF, sintered stainless-steel filter (Umetani Seiki Co., Model SLF); HLC, high-performance liquid chromatograph (Toyo Soda Co., Model HLC-803D) with a sample loop of internal volume of 300 μL; Co, helically coiled stainless-steel tube (0.2 mm (i.d.) × 2 m); G, pressure gauze (Umetani Seiki Co., Model LDG-50); GC, guard column (Toyo Soda Co., Model TSK gel GSWP, 7.5 mm (i.d.) × 150 mm); C_1, TSK gel G4000SW (7.5 mm (i.d.) × 600 mm); C_2, TSK gel G6000PW (7.5 mm (i.d.) × 600 mm); UMF, ultramembrane filter (Millipore filter with a pore size of 0.5 μm); LS, low-angle laser light scattering photometer (Toyo Soda Co., Model LS-8); DR, precision differential refractometer (Toyo Soda Co., Model RI-8); Re, double pen recorder; Dr, drain.

Cannan, 1936). Ovalbumin solution in 67 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.05% sodium azide, was filtered through a membrane filter (0.5 μm). Heat-induced ovalbumin aggregates were prepared as follows: 5 mL of 0.1% ovalbumin solution was put in a test tube with an internal diameter of 15 mm and immersed in a water bath kept at 30 °C. As soon as the solution was heated to a given temperature at the rate of 3 °C/min, the test tube was dipped into an ice–water mixture, and subsequent measurements were carried out at room temperature near 25 °C.

The ovalbumin solution thus prepared was applied to a high-performance gel chromatography system, consisting of a TSK gel G4000SW column (Toyo Soda Co.; 0.75 × 60 cm) and a TSK gel G6000PW column (Toyo Soda Co.; 0.75 × 60 cm), at a flow rate of 0.3 mL/min. Elution from columns was monitored with a low-angle laser light scattering photometer (LS-8, Toyo Soda Co.) and then with a precision differential refractometer (RI-8, Toyo, Soda Co.). The measuring system is schematically shown in Figure 1.

The molecular weight of heat-induced ovalbumin aggregates was estimated from the ratio of the output of a low-angle laser light scattering photometer, $(\text{output})_{LS}$, to that of a refractometer, $(\text{output})_{RI}$, by eq 1 (Takagi and Hizukuri, 1984; Maezawa et al., 1983), where k is a constant depending on the instrumental and experimental conditions and is determined by using standard protein. Native ovalbumin was used in this experiment as a molecular weight standard.

$$\text{MW} = k(\text{output})_{LS}/(\text{output})_{RI} \quad (1)$$

The weight-average molecular weight of the ovalbumin aggregates was determined by eq 2 (Takagi and Hizukuri, 1984), where $(\text{area})_{LS}$ and $(\text{area})_{RI}$ are the total areas in the elution peak of the LS photometer and the refractometer, respectively.

$$\text{MW} = k(\text{area})_{LS}/(\text{area})_{RI} \quad (2)$$

The heat-induced ovalbumin aggregates were treated with sodium dodecyl sulfate and dithiothreitol as follows: after the ovalbumin aggregates were formed at 80 and 90 °C, they were immediately cooled down to 25 °C, and then 0.5% sodium dodecyl sulfate and 0.02% dithiothreitol were added to 10 mL of 0.1% ovalbumin solution. The sample solutions (0.3 mL) thus prepared were applied to the high-performance gel chromatography system using the elution buffer containing 0.5% SDS or/and 0.02% dithiothreitol.

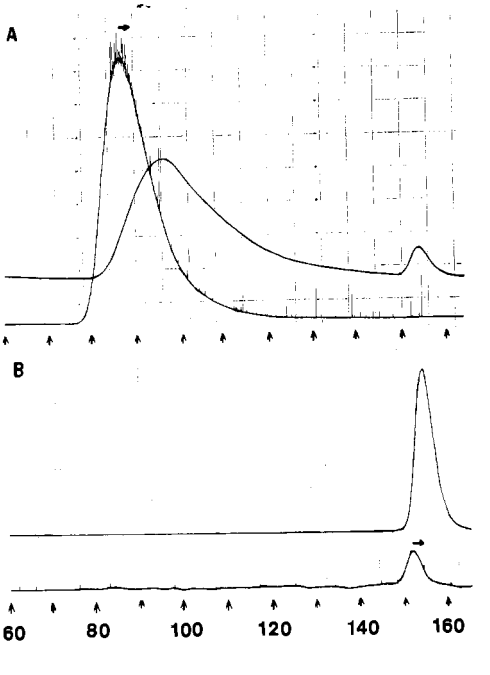

Figure 2. Elution patterns of ovalbumin aggregates (A) and native ovalbumin (B) obtained with a low-angle laser light scattering photometer (noisy curve) and a precision differential refractometer (smooth curve). Ovalbumin aggregates were formed by heat treatment up to 82 °C. The gain of LS photometer was set at 4 and that of refractometer at 16 for the ovalbumin aggregates. On the other hand, the gain of LS photometer was set at 32 and that of refractometer at 64 for native ovalbumin.

RESULTS

When heat-treated ovalbumin was applied to a TSK gel G3000SW column in a previous study (Kato et al., 1983), all of the aggregated ovalbumin was found to be eluted at the void volume. Therefore, no information was available concerning the molecular weight distribution, although only the weight-average molecular weight of the aggregates could be estimated. As a result of a series of preliminary experiments, a tandem array of a TSK gel G4000SW and a TSK gel G6000PW columns was found to be efficient to fractionate the ovalbumin aggregates: the former and the latter specialized in the fractionation of the aggregates of smaller size and those of larger size, respectively.

Figure 2 shows a typical example of elution patterns obtained with ovalbumin (A) heated up to 82 °C and with native ovalbumin (B). The peak of ovalbumin aggregates detected with a precision refractomenter emerged in the broad range of retention time, 80–140 min, suggesting broad distribution of molecular weight. On the other hand, the peak of native ovalbumin emerged in the position of retention time of 154 min. The gain setting of the light-scattering photometer for native ovalbumin was made eight times for ovalbumin aggregates, because the molecular weight of ovalbumin is too small to detect the peak of LS by using the same gain as heat-treated ovalbumin. Due to the difference of the positions of two cells along the flow line and two pens of the recorder, there is an apparent time lag in the tracings of the outputs of two detectors. Therefore, the tracing of the LS photometer must be

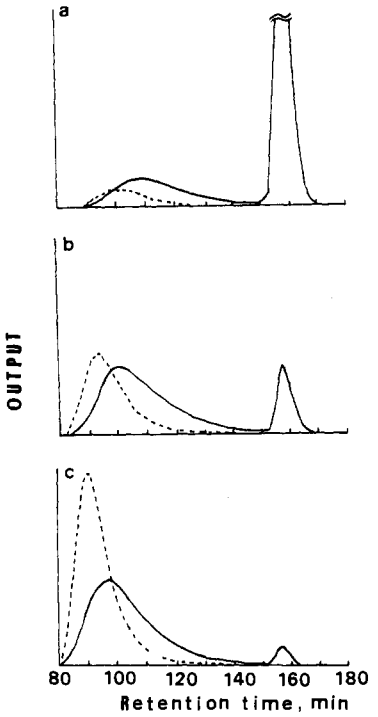

Figure 3. Changes in the elution patterns of ovalbumin aggregates heat treated up to 78 (a), 80 (b), and 82 °C (c) obtained with a low-angle laser light scattering photometer (---) and a differential refractometer (—). The gain of LS photometer was set at 4 and that of refractometer at 16.

shifted right in the distance indicated by an arrow shown in Figure 2. Even when the correction was made for the apparent time lag, it should be noted that the tracing of the scattering photometer is heavily biased to the left compared to that of refractometer. This clearly indicated that the ovalbumin aggregates are heavily populated by components with high molecular weight.

Figure 3 shows three pairs of the elution patterns of ovalbumin heat-treated up to various temperatures for which correction for the time lag and the inclination of the base lines has been made. The heat-induced ovalbumin aggregates in the experiments were obtained without insoluble aggregation. In addition, the recovery of the peaks eluted from the columns was 94, 96, and 91% in the heat-treated ovalbumin at 78, 80, and 82 °C, respectively. The recovery was calculated from the total area of the peaks of heat-treated ovalbumin compared with that for native ovalbumin. Thus, most ovalbumin aggregates seem to be recovered from the columns. Ovalbumin aggregates increased in amount in sacrifice of native ovalbumin with the heating temperature. On the other hand, the pair of peaks shifted to the side of shorter retention time with the heating temperature.

Figure 4 shows the change in the pair of elution patterns during prolonged standing at 25 °C after heat treatment up to 80 °C. Both the tracing of the light-scattering photometer and that of refractometer shifted to the side of shorter retention time. This demonstrates that, during prolonged standing at 25 °C, the ovalbumin aggregates formed at 80 °C heating proceed to further polymerization, while monomeric ovalbumin does not.

The weight-average molecular weight of the ovalbumin aggregates can be estimated from eq 2 described above. The weight-average molecular weights of the ovalbumin aggregates obtained in the experiments described above are compiled in Table I.

Figure 5 shows the effect of the addition of sodium dodecyl sulfate to the ovalbumin aggregates formed on

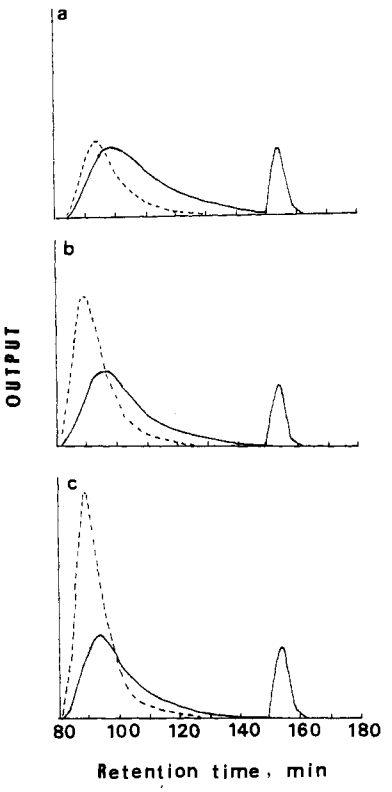

Figure 4. Changes in the elution patterns of ovalbumin aggregates during prolonged standing at 25 °C after heat treatment obtained with a low-angle laser light scattering photometer (---) and a differential refractometer (—). Ovalbumin aggregates were formed by heat treatment up to 80 °C, immediately cooled at 25 °C, and allowed to stand for 0 (a), 6 (b), and 22 h (c). The gain of LS photometer was set at 4 and that of refractometer at 16.

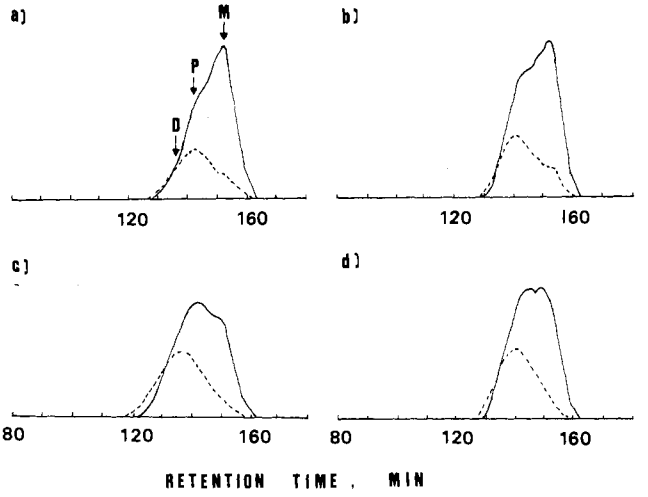

Figure 5. Effects of the addition of sodium dodecyl sulfate and dithiothreitol on the elution patterns of ovalbumin aggregates obtained with a low-angle laser light scattering photometer (---) and a differential refractometer (—): a, 0.5% sodium dodecyl sulfate was added to the ovalbumin aggregates formed on heat treatment up to 80 °C; b, 0.5% sodium dodecyl sulfate and 0.02% dithiothreitol were added to ovalbumin aggregates formed on heat treatment up to 80 °C; c, 0.5% sodium dodecyl sulfate was added to the ovalbumin aggregates formed on heat treatment up to 90 °C; d, 0.5% sodium dodecyl sulfate and 0.02% dithiothreitol were added to the ovalbumin aggregates formed on heat treatment up to 90 °C. Key: M, monomer (153 min); P, pentamer (142 min); D, decamer (135 min). The gain of LS photometer was set at 32 and that of refractometer at 64.

heat treatment up to 80 °C. The heat-induced ovalbumin aggregates were completely dissociated into monomeric and oligomeric forms by the addition of sodium dodecyl

Table I. Degrees of Polymerization (dp) of Ovalbumin Aggregates

ovalbumin aggregate	av dp[a]	distribn of dp[b]
heat induced at 78 °C	60	10–350
heat induced at 80 °C	130	15–730
heat induced at 82 °C	215	25–1000
room temperature[c]		
6 h	210	30–1000
22 h	260	50–1380

[a] Calculated from the average molecular weight of total peaks of ovalbumin aggregates. [b] Calculated from Figures 6 and 7. [c] Ovalbumin aggregates heat induced at 80 °C were cooled at 25 °C and then allowed to stand for 6 and 22 h.

sulfate. The degree of polymerization was mostly less than 10, as shown in the figure. It should be noted that monomeric ovalbumin is the major component. Further addition of dithiothreitol had no appreciable effect on the elution pattern. When ovalbumin had been heat treated up to 90 °C, the increase in the degree of polymerization of oligomers was observed after the addition of SDS. In this case, further addition of dithiothreitol decreased the population of the oligomers. This reflects a partial formation of disulfide bonds by heating up to 90 °C.

DISCUSSION

Superiority of the Present Technique. In the previous paper (Kato et al., 1983), we have for the first time introduced to the study of the formation of heat-induced aggregates of protein the use of a measuring system where proteins are separated by high-performance gel chromatography and then monitored with respect to molecular weight by a low-angle laser light scattering photometer and a differential refractometer. Due to the small pore size of the gel of the prepacked column, TSK gel G3000SW, all the ovalbumin aggregate was eluted at the void volume to allow only the estimation of weight-average molecular weight of the total aggregates. As is clear from Figures 2–4, the introduction of a TSK gel G6000PW column usually used for polysaccharides with wide molecular weight distribution and a TSK gel G4000SW instead of G3000SW made possible the fractionation of the ovalbumin aggregates. The technique used in the present study allowing the estimation of molecular weight distribution within a couple of hours is unrivalled by the sedimentation equilibrium technique, the only alternative to the present technique, which is not only time-consuming but also low-resolving with respect to molecular weight distribution.

Stepwise Nature of the Aggregation. All the elution patterns in Figures 2–4 indicate that there are highly aggregated ovalbumins and a monomeric one and that the population of the aggregates of oligomeric size is very low. When treated with sodium dodecyl sulfate, the aggregates were disassembled to give a mixture of oligomeric aggregates and monomers of ovalbumin. It is very probable, therefore, that the heat-treated ovalbumin molecules first form oligomeric aggregates and they further rapidly aggregate to give the highly associated aggregates with wide molecular weight distribution. To our surprise, disaggregation could be brought about only by treatment of sodium dodecyl sulfate effective to disrupt noncovalent interactions. The observation is against the general concept that the aggregate formation of proteins on heat treatment is mostly the result of formation of intermolecular disulfide bonds (Van Kleef et al., 1978; Van Kleef, 1986; Utsumi et al., 1984). Judging from the result of the presence of dithiothreitol, disulfide bonds only partially play a role in the aggregate formation of ovalbumin heat-treated up to 90 °C. The above result makes it necessary to carefully reassess the importance of disulfide bonds in the intermolecular association during heat denaturation of proteins.

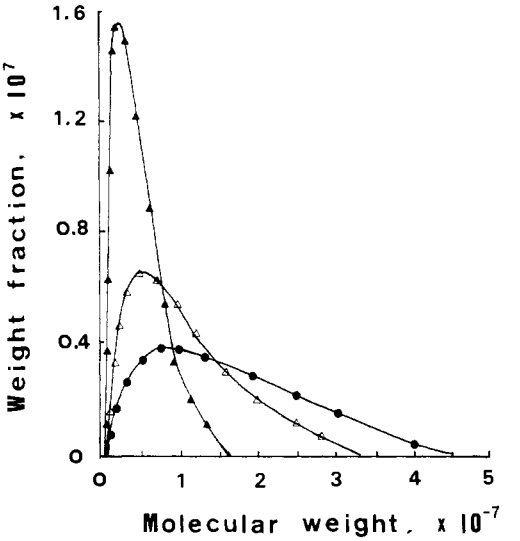

Figure 6. Molecular weight distribution curves of the ovalbumin aggregates formed on heat treatment up to 78 (▲), 80 (△), and 82 °C (●).

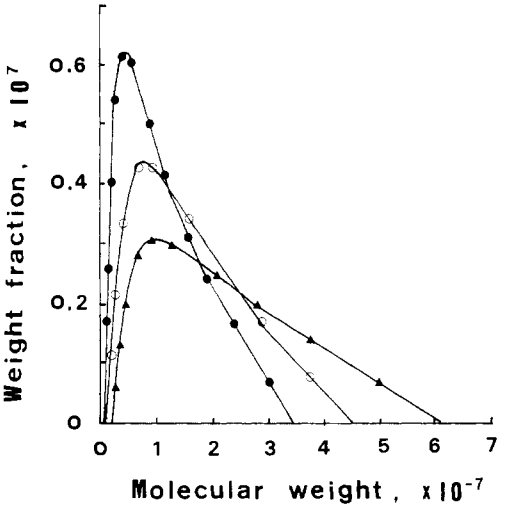

Figure 7. Molecular weight distribution curves of the ovalbumin aggregates formed on standing at 25 °C for 0 (●), 6 (○), and 22 h (▲) after heat treatment up to 80 °C.

Molecular Weight Distribution. The elution curves in Figures 3 and 4 make it possible to estimate the molecular weight of the fraction eluted at a particular retention time from eq 1 and its relative population from the output of the refractometer. Each pair of elution curves in Figures 3 and 4 can be converted to a corresponding molecular weight distribution curve. The molecular weight distribution curves of ovalbumin aggregates were drawn according to eq 3, where $f(M)$ is a weight fraction of the

$$f(M) = k(\text{output})_{RI} \quad (3)$$

component with a particular molecular weight, k is a constant, and $(\text{output})_{RI}$ is the output of refractometer when the component was eluted. The molecular weight was hard to determine at the extreme outskirts of the elution curves in Figures 3 and 4. Therefore, they were determined by extrapolation. The distribution curves were subsequently normalized according to eq 4. The distribution curves thus obtained were shown in Figures 6 and

$$k\int_0^\infty (\text{output})\, dM = 1 \quad (4)$$

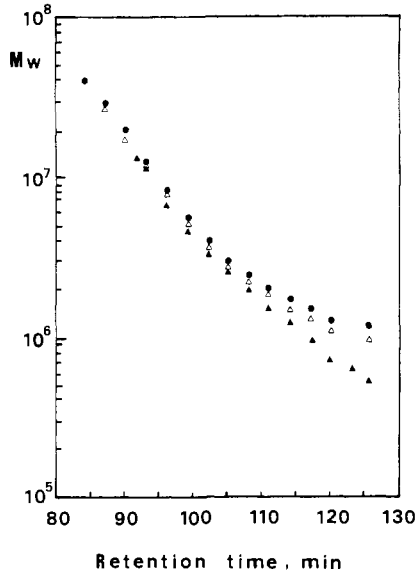

Figure 8. Relationship between molecular weight and retention time of the ovalbumin aggregates formed on heat treatment up to 78 (▲), 80 (△), and 82 °C (●).

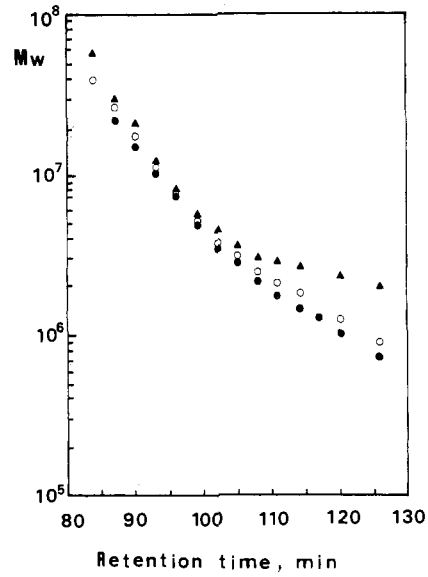

Figure 9. Relationship between molecular weight and retention time of the ovalbumin aggregates formed on standing at 25 °C for 0 (●), 6 (○), and 22 h (▲).

7. Figure 6 shows the molecular weight distribution curves of ovalbumin aggregates heat-treated at 78, 80, and 82 °C. The distribution curves of ovalbumin aggregates are shifted to the side of higher molecular weight and become flatter, suggesting a rapid progress of aggregation with the heating temperature. In the heating system used here, different heating temperature involves different heating time at the same time. Therefore, the heating time also should be considered as a factor of the progress of aggregation. Figure 7 shows the changes in the molecular weight distribution curves during prolonged standing at 25 °C after heat treatment up to 80 °C. The distribution curves of ovalbumin aggregates are shifted to the side of higher molecular weight and become flatter, suggesting further progress of aggregation at room temperature.

Effect of Temperature and Aging on the Hydrodynamic Property of the Aggregates. Figures 8 and 9 show the plots of molecular weight vs. retention time of ovalbumin aggregates. These curves were obtained by converting the elution curves in Figure 3 and 4. The curves are shown here to emphasize the effect of temperature of heat treatment and time of standing at room temperature on the nature of the aggregates. As shown in Figures 8 and 9, the molecular weight of the fraction eluted at a particular retention time increased with the temperature of heat treatment and the time of standing at room temperature. This indicates that not only do the aggregates increase their degree of association but they become more compact with the changes in these parameters, if the retention time reflects the size of the aggregates eluted at a particular retention time.

Conclusion. Heat-induced ovalbumin aggregates with a wide range of molecular weight distribution were efficiently fractionated by high-performance gel chromatography using a tandem array of TSK gel G4000SW and TSK gel G6000PW. The initial phase of heat denaturation of proteins with respect to the aggregation was elucidated from the elution patterns detected with a low-angle laser light scattering photometer and a differential refractometer. The results obtained seem to give insight into the unknown part of the heat denaturation of proteins. The results suggesting the formation of a more compact structure may reflect that a kind of regular structure is formed with aging of the heat-induced aggregates.

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