Laser Light Scattering Study on the Heat-Induced Ovalbumin Aggregates Related to Its Gelling Property

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A Multiangle laser photometer and a dynamic light scattering spectrophotometer were used to characterize the aggregation behavior and gel network structure of elastic and less elastic gels of ovalbumin. Ovalbumin formed opaque and less elastic gels in 50 mM phosphate buffer (pH 7.0). However, succinylated ovalbumin formed transparent and elastic gels. Heat-induced ovalbumin protein formed high molecular weight and polydisperse aggregates. Succinylation of the protein contributed to formation of lower molecular weight polymers with a narrow molecular size distribution during subsequent heat gelation. The root mean square and hydrodynamic radii of the protein polymers indicated that the elastic gel consisted of a more extended protein polymer network compared to that of the less elastic gel. The aggregation behavior of denatured ovalbumin appeared to play a crucial role in the subsequent gelling process of the proteins.

Keywords: *Ovalbumin; light scattering; aggregate; gelation; succinylation; elastic gel; molecular size*

INTRODUCTION

Elucidation of the protein structure−function relationship in food proteins can result in useful practical information in food and protein chemistry. Egg white proteins display multiple functional properties such as foaming and gelling. Although numerous methods exist to study protein structure, only limited information is available concerning the relationship between protein structure and functionalities of food proteins. In general, a protein gel network is formed via noncovalent cross-linkages such as hydrophobic interactions, or electrostatic and hydrogen bond interactions, and less frequently by covalent interactions such as disulfide bonds to form a three-dimensional network (Ziegler and Foegeding, 1990; Clark, 1992). Two types of gels are commonly found in food (Clark, 1992). Ovalbumin gels formed at pH values between 4 and 6 are turbid, while gels made at pH values above or below this range are clear (Hatta *et al*., 1986). The microstructure of pH 5 gels (turbid) is granular and inhomogeneous compared to the uniform homogeneous appearance of a pH 10 gel (clear) (Heertje and Van Kleef, 1986). Gel hardness and rigidity are also related to NaCl (Egelandsdal, 1980). Transparent gels obtained by two-step heating methods were firm and elastic with high-water-holding capacity, while the opaque gel obtained by one-step heating was soft and less elastic (Kitabatake *et al*., 1989). It was also reported that a particular networks of proteins was formed when partially unfolded ovalbumin aggregate randomly into grapelike clusters (turbid gel) or linearly to form a "string of bead" (clear gel) which is dependent on pH, ionic strength, and protein concentration (Doi, 1993). Furthermore, heat-induced ovalbumin protein forms a transparent solution by the succinylation of the amino groups in the protein (Nakamura *et al*., 1978; Ma and Holme, 1982). It is believed that the gelling properties of proteins could be affected by factors determining the state of aggregates found during the subsequent heat for gelation. However, there is little information regarding the comparative studies on the aggregation behavior polymer size, and hydrodynamic radius between elastic gels and less elastic ones. Succinylated ovalbumin is suitable for the elucidation of the aggregation mechanism of proteins, because the repulsive electrostatic interactions, which may affect the physical characteristics of protein gels, would be changed by the modification. Absolute characterization of these protein aggregates is neccessary to understand these phenomena. Thus, it is of great interest to elucidate the relationship between gelling properties of ovalbumin upon heat-induced gelation and the aggregation behavior of elastic and less elastic gels.

The development of laser light scattering techniques has enabled advances in polymer science for characterizing the aggregation behavior and net work structure from polymer gels. Light scattering is an ideal means by which to determine the actual molecular weight and whether any aggregates have formed (Wyatt, 1993; Shortt, 1993). In this study, the author demonstrates that the technique is a useful tool in characterizing protein aggregation behavior and gel structure upon heating *in situ*. Multiangle laser scattering coupled with size-exclusive HPLC and dynamic light spectrophotometry allows us to obtain not only an absolute molecular weight and the root mean square (rms) radius of protein polymers but also the hydrodynamic radius of the protein gel network *in situ*.

MATERIALS AND METHODS

Materials. Ovalbumin was purchased from Sigma Chemicals, St. Louis, MO. Other chemicals used were ACS grade from Sigma. Ovalbumin was succinylated according to the procedure described by Groninger (1973). Succinic anhydride was added at a 50:1 (protein/anhydride, weight ratio). The extent of succinylation was determined from the free amino contents by the method of Concon (1975) using dinitrobenzenesulfonate (DNBS).

Preparation of Gels. A protein solution (8.0%) in 50 mM phosphate buffer (pH 7.0) was injected into an aluminum cup (5 mm inner diameter, 2.5 mm height). The cup was filled, completely degassed, and sealed with a silicone sheet on which a steel plate was tightly fixed with clips. The cups containing protein solution were heated at 80 °C for 20 min, followed by rapid cooling to room temperature by immersion in tap water.

Creep Analysis of Gels. Creep behavior under compression was analyzed with a Rheoner RE-3305 (Yamaden, Co.

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Ltd., Tokyo) interfaced with a computer (NEC PC 9801 VM, NEC, Tokyo). The creep curves were analyzed according to the procedure described by Kamata *et al*. (1988).

Light Scattering Measurements. Heat-induced ovalbumin protein aggregates were prepared as follows; Diluted protein solution (0.1% protein in 10 mM phosphate buffer, pH 7.5) was passed through a membrane filter (0.45 μm, Waters) with subsequent adjustment of the protein concentration spectrophotometrically. A 3 mL aliquot of the protein solution was put into a test tube with a screw cap, and the tube was immersed in a water bath at 80 °C for 20 min and then cooled at room temperature, immediately. The sample (200 μL) was used for the multiangle laser light scattering (MALLS) experiments. Light scattering was measured on a DAWN DSP-F MALLS (Wyatt Technology, Santa Barbara, CA) using a 632.8 nm laser and dynamic light scattering spectrophotometer (DLS-7000, Otsuka Electronics, Tokyo). The MALLS photometer was coupled with a HPLC system consisting of Shodex KW-804 and KW-803 columns at room temperature. A specific refraction index increment (dn/dc) of 0.186 was obtained for the dialyzed protein solution using a Wyatt Optilab DSP interferometric differential refractometer. The second virile coefficient of 1.94×10^{-4} was calculated from a Zimm plot. Data were collected and processed by the DAWN data collection program.

Equations. The MALLS photometer measures the intensity of the scattered light with the use of photodiodes placed at specific angles (θ) relative to the laser beam. This light intensity is converted to the Rayleigh ratio as

$$R_\theta = I_\theta r^2/(I_0 V) \quad (1)$$

where R_θ is the Rayleigh ratio, I_θ is the scattered intensity, I_0 is the incident beam intensity, V is the scattering volume, and r is the distance between scattering volume and detector.

From the Rayleigh ratio, we can use the Zimm equation (eq 2) to directly determine molecular weight provided that a number of physical properties are known

$$R_\theta/K^*c = MP(\theta) + 2A_2cM^2P^2(\theta) \quad (2)$$

where R_θ is the Rayleigh ratio, K^* is an optical constant, $4\pi^2 n_0^2(dn/dc)^2\lambda_0^{-4}N_A^{-1}$ (where n_0 is the refractive index of the solvent at the incident radiation wavelength (nm), N_A is Avogadro's number and dn/dc is the differential refractive index increment of the solvent−solute solution with respect to a change in solute concentration), c is the concentration of solute molecules in the solvent, $P(\theta)$ is the theoretically derived form factor ($P(\theta)$ is a function of the molecule's z-average size, shape, and structure), A_2 is the second virial coefficient, and M is the weight-average molecular weight.

Since we are using multiangle detection here, we can extrapolate the value of θ to 0. In the limit of $\theta \rightarrow 0$, $P(\theta) \rightarrow 1$ and eq 2 becomes

$$R_{\theta\rightarrow 0}/K^*c = M + 2A_2cM^2 \quad (3)$$

If $A_2 = 0$, then

$$M = R_\theta/K^*c \quad (4)$$

Using the Debye plot

$$R_\theta/K^*c \text{ vs } \sin^2(\theta/2) \quad (5)$$

We can see that at an angle of $\theta = 0$, we can read the molecular weight (M) directly off of the y-axis of the plot. It depends on the rms radius $\langle r^2\rangle$ independent of molecular conformation (Wyatt, 1993).

Using a *continuum model* (Tanaka, 1985) where the wavelength of light is usually much larger than the average distance between neighboring cross-links, a self-correlation function is derived as the linear equation

$$C(\tau) \sim \alpha(kT/K)\exp(-Dq^2\tau)$$

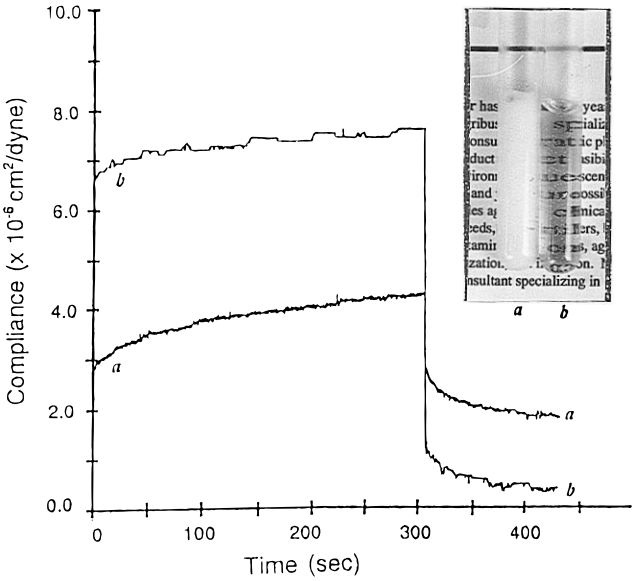

Figure 1. Creep and creep−recovery curves of (a) ovalbumin and (b) Su-ovalbumin gels. The protein solution (8.0% in 50 mM phosphate buffer, pH 7.0) was heated at 80 °C for 20 min, followed by rapid cooling to room temperature by immersion in tap water.

where $C(\tau)$ is the self-correlation function, k is the Boltzmann constant, T is the absolute temperature, K is the modulus of elasticity, q is the scattering vector, and D is the diffusion coefficient.

We produce the Stokes−Einstein relation for the diffusion coefficent of polymers in solution

$$D = kT/6\pi\eta_0 a$$

where η is the liquid viscosity and a is the hydrodynamic radius of the polymer.

RESULTS AND DISCUSSION

Creep Analysis of Ovalbumin Gels. By using succinic anhydride, 28% of the lysine of ovalbumin was modified (data are not shown). The gel formation of the succinylated ovalbumin (Su-ovalbumin) protein was compared to that of ovalbumin. Ovalbumin formed an opaque gel at pH 7.0 upon heating at 80 °C for 20 min in 50 mM phosphate buffer, pH 7.0. On the other hand, Su-ovalbumin gel gave a transparent gel under the same conditions (inset of Figure 1). The typical creep and creep−recovery curves of these two gels were obtained to clarify the difference in the gel characteristics of a transparent and an opaque gel. The creep compliance of the transparent gel was more than twice that of the opaque gel, indicating that the transparent gel was more soft and deformable. Differences in the rheological properties of theses gels were also observed clearly by the residual strain after the removal of the stress. The Su-ovalbumin gels showed lower residual strain than ovalbumin gels (Figure 1). These results suggested that the Su-ovalbumin gels were more elastic, while those of ovalbumin were likely to be more plastic or less elastic. Creep curves obtained were further analyzed with a four-element mechanical model as shown in Table 1. Each parameter (P, stress; E_0, instantaneous modulus; E_1, retarded elastic modulus; η_1, retarded viscous modulus; and η_N, Newtonian viscous modulus) was calculated. The P and E_0 values of the ovalbumin gels were over twice than that of the Su-ovalbumin gels, while E_1, η_1, and η_N values were smaller in the opaque gels. These results corresponded to transparent gels having a smaller instaneous deforma-

Table 1. Viscoelastic Parameters of Ovalbumin and Su-ovalbumin Gels

parameters[a]	ovalbumin	Su-ovalbumin
P ($\times 10^4$)	4.99	2.50
E_0 ($\times 10^5$)	3.57	1.52
E_1 ($\times 10^6$)	1.46	2.05
η_1 ($\times 10^7$)	4.86	3.31
η_N ($\times 10^8$)	4.00	6.08
τ_1	33.19	16.13

[a] P, stress; E_0, instantaneous modulus; E_1, retarded elastic modulus; η_1, retarded viscous modulus; η_N, Newtonian modulus.

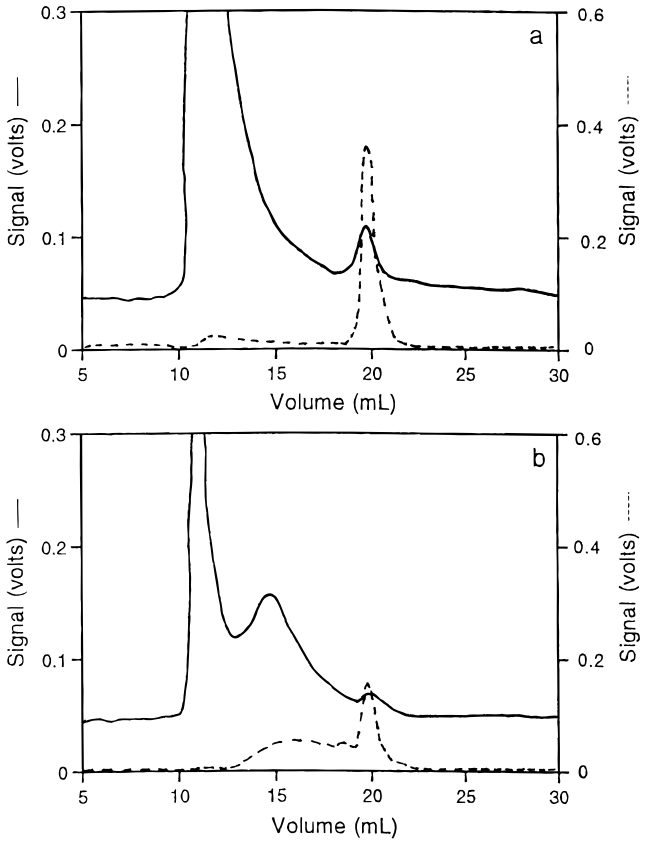

Figure 2. Elution patterns of (a) ovalbumin and (b) Su-ovalbumin aggregates obtained with a MALLS system. Solid line, results from DAWN F 90° detector output; broken line, results from RI signal. The protein solution (0.1% in 10 mM phosphate buffer, pH 7.5) was heated at 80 °C for 20 min and then cooled to room temperature, immediately. Column sample size, 200 μL.

tion but a larger retarted deformation, with time up to 300 s, than the opaque gels. The results suggested that the Su-ovalbumin gels were more elastic, while the opaque gels were more viscous.

Laser Light Scattering. It is well-known that the gelling properties may be affected by the cross-linking of unfolded molecules as a result of hydrogen bonding and ionic and hydrophobic interactions (Damodaran, 1994). The turbidity of the ovalbumin gels depends on the pH and ionic strength of the heated protein solution (Hatta *et al.*, 1986). In the past, low-angle laser light scattering was used to estimate the molecular weight distribution of heat-induced ovalbumin and dry-heated egg white protein aggregates (Kato and Takagi, 1987; Kato *et al.*, 1990). However, this system has been hampered severely by solvent noise due to impurities. The MALLS system reduces dramatically the effect of noisy background, giving cleaner data and highly reproducible results (Wyatt, 1993). Figure 2 shows the DAWN F 90° detector output and RI signal for the heat-induced aggregates of ovalbumin and Su-ovalbumin. The MALLS system clearly detected a large aggregate at 10 mL that was almost invisible to the RI detector. Interestingly, the aggregation peak of Su-ovalbumin showed two spread peaks at volumes of 10 and 12.5 mL. This indicates that the aggregation behavior of Su-ovalbumin was different from ovalbumin. The molecular weight and rms of heat-induced soluble aggregates were analyzed by the MALLS system. Figure 3 shows the relationship between retention volume (HPLC) and molecular weight of heat-induced aggregates of the proteins. There was a remarkable reference for both aggregates of molecular weight vs volume. Ovalbumin aggregates showed molecular weights between 1.7×10^6 and 1.6×10^8, while the range for Su-ovalbumin was lower, being between 2.1×10^5 and 4.2×10^7. In general, the retention volume of HPLC gel filtration column reflects the size and mode of proteins eluted at a particular retention volume. Figure 4 shows the relationship between the rms radius and the retention volume (HPLC) of the heat-induced aggregates of the proteins. Interestingly, there was little difference in the rms radius for both the aggregates except at high retention volume, in spite of the considerable differences in the absolute molecular weight.

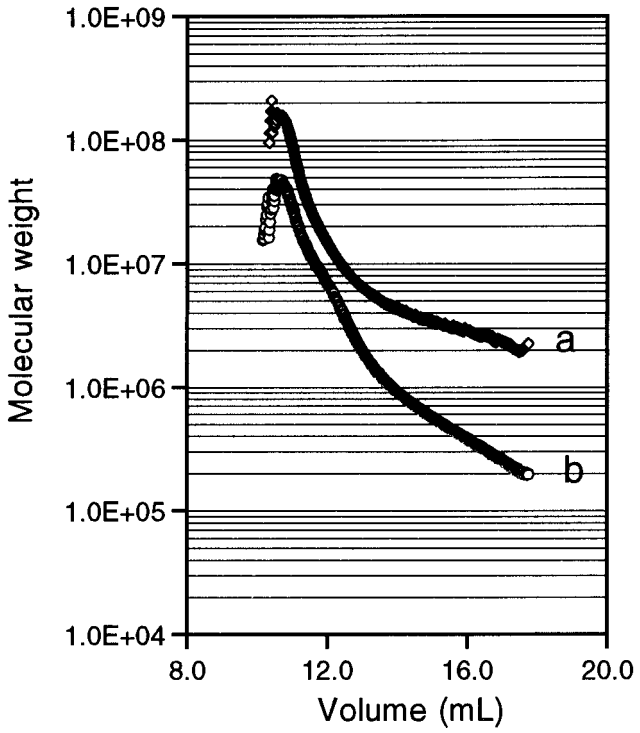

Figure 3. Relationship between absolute molecular weight and retention volume (HPLC) of (a) ovalbumin and (b) Su-ovalbumin soluble arregates. Conditions are the same as in Figure 2.

Table 2 summarizes the laser light scattering characteristics of the heat-induced ovalbumin and Su-ovalbumin aggregates. The data indicated that the molecular weight distribution of the heat-induced ovalbumin aggregates was large and heterogeneous. By contrast, that of the Su-ovalbumin was smaller and less heterogeneous. Interestingly, no significant differences in the rms of the aggregates between ovalbumin and Su-ovalbumin were shown. These results suggest that succinylation of ovalbumin contributes to the formation of lower molecular weight and narrower molecular size distribution in the aggregates found upon heating. The formation of extended structures of heat-induced Su-

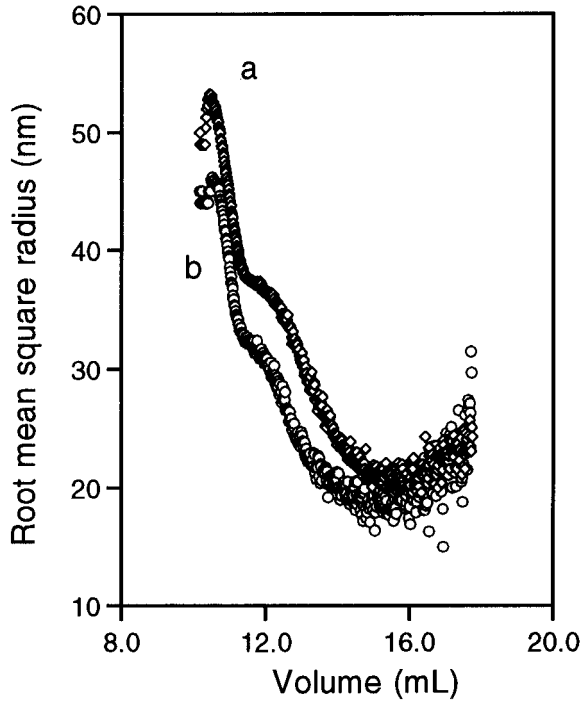

Figure 4. Relationship between rms radius and retention volume (HPLC) of (a) ovalbumin and (b) Su-ovalbumin soluble arregates. Conditions are the same as in Figure 2.

Table 2. Laser Light Scattering Characteristics of Heat-Induced Ovalbumin and Su-ovalbumin Aggregates[a]

samples	M_w ($\times 10^4$)[b]	M_n ($\times 10^4$)[c]	M_w/M_n	rms (w)[d]
ovalbumin	322.60	9.24	34.91	25.2
Su-ovalbumin	59.35	10.01	5.93	23.9

[a] A 0.1% protein sample in 50 mM phosphate buffer (pH 7.0) was heated at 80 °C for 20 min. [b] Weight-average molecular weight (g/mol). [c] Number-average molecular weight (g/mol). [d] rms radius (nm).

ovalbumin polymer was also predicted from the rms data for the aggregates. Similar results were observed in the case of dry-heated egg white protein, which showed excellent gel strength (Kato *et al.*, 1990; Mine, 1996). Thus, the aggregation behavior of denatured proteins appears to play a crucial role in the subsequent gelling process of proteins.

The mean diameter and distribution of the diameters of the gel matrix obtained by a dynamic light scattering system is shown in Figure 5. As shown in Figure 5, the diameter distribution of the gel matrix in the histogram was spread widely. The ovalbumin gel matrix gave three spread distribution peaks and that of Su-ovalbumin gave two. This indicates that the theoretical approch of the *continuum model* shows ideal conditions for a gel matrix; however, the actual gel matrix of protein polymers is more complicated. The smaller peak 1 for ovalbumin gel may be caused by dangling chains which are not linked completely the gel matrix. The hydrodynamic radius of the Su-ovalbumin polymer was larger than that in the ovalbumin gel. These data indicate that an elastic gel is composed of a firm polymer network but a wide space gel matrix. An opaque (less elastic) gel is made from a heterogenous aggregate gel network. The number of elastically active chains may increase with increasing transparency of the gel. Hydrophobic interactions and repulsive electrostatic interactions affect the physical characteristics of protein gels (Damodaran, 1994). The aggregation behavior of Su-ovalbumin may be caused by an increase

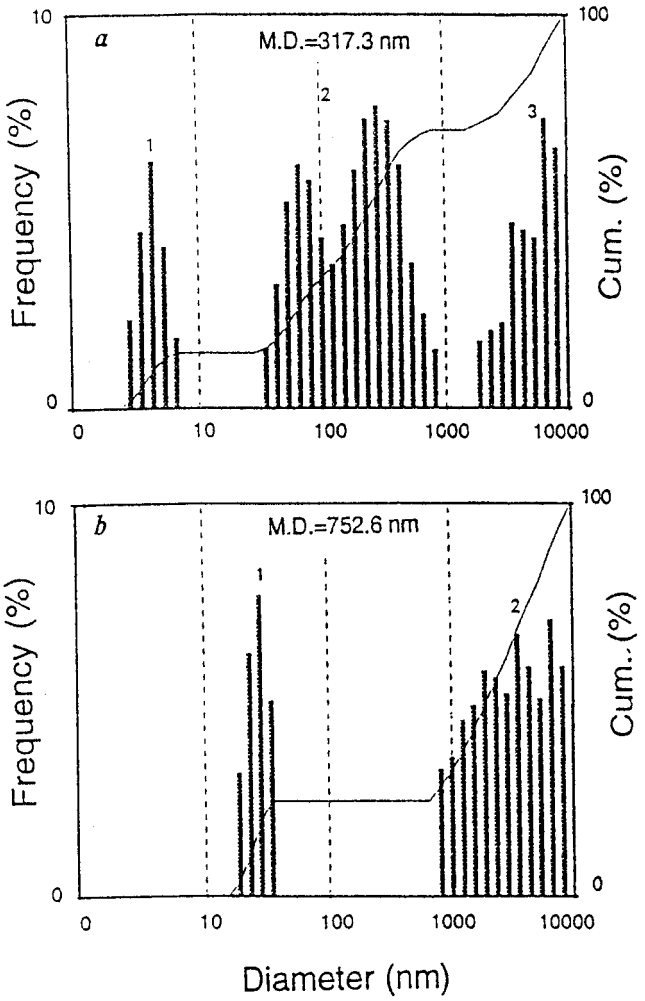

Figure 5. Particle diameter distributions for (a) ovalbumin and (b) Su-ovalbumin gels. Protein solution (1 mL) (8.0% in 10 mM phosphate buffer, pH 7.5) was placed in the measuring glass cell (diameter, 5 mm) and heated at 80 °C for 20 min after degassing. The data were obtained by use of a dynamic light scattering system. M.D., mean diameter; Cum, accumulation.

in the electrostatic repulsion by chemical modification and may be related to the subsequent formation of elastic gels.

Structual changes in protein molecules between elastic and less elastic gels are also of great interest in food systems. However, fundamental information on this issue is limited because of the difficulty in analyzing solidlike structures at high protein concentration. Ovalbumin that has received sufficient heat treatment to aggregate retains substantial secondary structure and is slightly less compact than the native molecule (Kato and Takagi, 1988). Raman spectroscopy indicated that ovalbumin appeared to be aggregating via an intermolecular β-sheet structure (Painter and Koening, 1976), and the β-sheet structure increased with a simultaneous decrease of helical structure in heated transparent gels of α-lactalbumin (Nonaka *et al.*, 1993). However, Ozaki *et al.* (1992) reported that most of the α-helix structure changed into an unordered structure in the proteins constituting egg white upon heating using near-infrared FT-Raman spectroscopy. Combination of the laser light scattering technique and protein structual analysis using for example FT-Raman spectroscopy *in situ* would be desirable for further investigation.

Conclusion. This study demonstrated that multi-

angle laser light scattering is a useful tool for analyzing the heat-induced aggregation behavior of proteins and the network structure of protein polymers *in situ*, which may be valuable for correlation with protein functionality in food systems. In this work, it was indicated that elastic gels consisted of lower molecular weight and more extended protein polymers, which were found during subsequent heat gelation, and a wide space network with transparency. However, less elastic gels were composed of higher molecular weight aggregates. The aggregation behavior of denatured proteins appears to play a crucial role in the gelling process of proteins.

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