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Structural and Gelling Properties of Dry-Heating Egg White Proteins

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Gel strength of dried egg white greatly increased by heating in the dry state at 80 °C. The structural changes responsible for the enhancement of gel strength were studied. Heat-induced gelation of dry-heated egg white in the presence of denaturing reagents resulted in a decrease of gel strength in the order SDS > urea > 2-mercaptoethanol. CD spectra revealed only little changes in the secondary structure of some egg white proteins upon dry-heating, except ovotransferrin. Proteolytic digestibility of ovalbumin and ovotransferrin increased progressively with an increase of dry-heating time, indicating enhancement of protein flexibility by such heating in the dry state. DSC thermograms showed that enthalpy of denaturation (ΔH) of dried egg white was markedly decreased with an increase of dry-heating time. In parallel, the Gibbs free energy of unfolding in water (ΔG) was also found to decrease at approximately the same rate as ΔH. A good correlation was obtained between the decrease in ΔH and the increase in gel strength of egg white proteins heated in the dry state.

Dry-heating is required for the manufacture of processed foods and the reduction of their microbial population. These processes could cause substantial protein denaturation, which is critical to functionalities such as solubility, gelation, emulsification, and foaming (Kinsella, 1976). Our previous report (Kato et al., 1989) showed that when spray-dried egg white was heated in a controlled dry state at 80 °C for various periods of time, its functionalities (gelling, emulsifying, and foaming properties) improved significantly with an increase in heating time without loss in solubility. However, the molecular basis of structural changes causing the improvements of functional properties in dry-heating egg white has yet to be unraveled. Furthermore, there is little information about the dry denaturation of proteins, despite the importance to industrial applications.

The present paper describes the conformational changes in spray-dried egg white proteins by dry-heating treatment and the relationship between the structural and functional properties, especially gel formation.

MATERIALS AND METHODS

Dried egg white (DEW) was prepared by spray-drying at 70 °C after decarbohydrate treatment. Ovalbumin was prepared from fresh egg white by a crystallization method in sodium sul-

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fate and recrystallized five times. Ovotransferrin was prepared from fresh egg white, as described by Williams et al. (1962). All reagents used were of analytical grade.

Heat treatment of all samples used in this study was done in the dry state in an incubator at 80 °C in a given period (days) and then immediately cooled to room temperature.

Circular dichroism (CD) analysis was carried out to assess the conformational changes of DEW heated in the dry state. Protein samples (10 mg) were dissolved in 7 mL of 10 mM sodium phosphate buffer, pH 7.2. Each of samples was filtered with an Ekicorodisk filter (pore size 0.22 μm). The filtrate was used for CD analysis. CD spectra were measured on a Jasco Model J-500 spectropolarimeter in the far-ultraviolet region (190–260 nm) with a 0.009-cm path length cell at 25 °C. The accuracy of the data was improved by averaging eight scans integrated with the data processor (Model DP-501). CD spectra were represented in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$).

Protease digestibility was determined by using chymotrypsin. The enzymatic reaction was carried out at 38 °C for a given time with a protein to enzyme ratio of 16:1. To 3 mL of 0.1% protein solution in 50 mM Tris-HCl buffer, pH 8.0, was added 200 μL of 0.1% chymotrypsin solution, and proteolysis was monitored by the trichloroacetic acid (TCA) precipitation method. A 3-mL portion of 4.0% TCA was added to terminate the enzymatic reaction and precipitate the undigested protein. The resultant mixture was allowed to stand for 10 min and filtered (Toyo Roshi Ltd., No. 5b), and the absorbance of TCA-soluble peptides in the filtrate was measured at 280 nm on a spectrophotometer to estimate the amount of digest in the filtrate. The extent of digestion was expressed as the percentage of absorbance of 0.05% untreated sample. The digestion velocity was represented as the percent digested in 1 min, calculated from the initial slopes of digestion curves against time.

The thermal characteristics of egg white proteins heated in the dry state for various periods were examined by differential scanning calorimetry (Seiko, DSC-100 thermal analyzer, equipped with a DSC cell). In a typical experiment, 50 μL of 10% protein solution was sealed in a preweighed hermetic aluminum pan and the pan with its contents was weighed again. Another pan containing water with no protein was used as the reference pan. The pans were then heated in the calorimeter at a linear rate of 1 °C/min over the range 30–120 °C. The denaturation temperature (T_d) and enthalpy of denaturation (ΔH) were computed from the thermograms by the SSC-5000 analyzer (Seiko Electric Industry Co.).

The Gibbs free energies of unfolding (ΔG) of DEW and ovalbumin heated in the dry state were determined by measuring the CD spectra of 0.1% protein solutions at various urea concentrations. The apparent fraction of unfolding (f_{app}) was calculated by $f_{app} = ([\theta]_n - [\theta])/([\theta]_n - [\theta]_d)$, where $[\theta]$ represents the negative ellipticity at 222 nm at a given concentration of urea and $[\theta]_n$ and $[\theta]_d$ represent the negative ellipticity at 222 nm in the absence and the presence of 10 M urea, respectively. The equilibrium constant for unfolding, K, was calculated by $K = f_{app}/(1 - f_{app})$, and the K value in the absence of urea was estimated by extrapolation of the linear K vs urea concentration plot. Gibbs free energy of unfolding in water (ΔG) was calculated from

$$\Delta G = -RT \ln K$$

where R is the gas constant, T is the absolute temperature, and K is the equilibrium constant for unfolding in water.

The thermal gelation of dried egg white proteins was done by the method described previously (Kato et al., 1989). Protein solutions in water with different concentrations were heated at 80 °C for 20 min and then cooled to about 25 °C. Gel strength of the heated samples was measured with an tensile tester (Tensilon/UTM-II, Toyo Baldwin Co.). The gel strength was expressed as breaking stress (g) was exerted by a cylindrical plunger with a flat section (0.5-cm diameter).

RESULTS AND DISCUSSION

The dependency of gel strength on the protein concentration of DEW heated in the dry state is shown in Figure 1. The gel strength of DEW heated in the dry state for different lengths of time markedly increased with an increase in protein concentration. The effect of heating time in the dry state on gel strength was more pronounced at 10% protein concentration than at lower concentrations. The gel strength of DEW greatly increased with an increase in heating time in the dry state. The dependency of gel strength upon protein concentration was substantial for samples heated in the dry state compared to that of native DEW. This result indicates that the gel strength of DEW is dependent on protein concentration. Also it suggests that heating in the dry state is an effective method to reduce the amount of protein required to yield a gel with particular gel strength. For example, a gel with a hardness of 30 g could be obtained from a 7% concentration of DEW heated for 10 days, while this gel strength could result from a 10% concentration of native DEW.

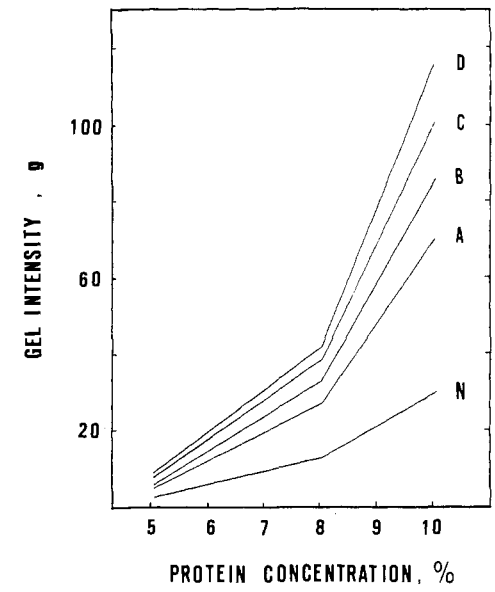

Figure 1. Effect of protein concentration on gel strength of heated DEW in the dry state for various periods of time: N, nonheated sample; A–D, heated samples for 3, 5, 7, and 10 days, respectively.

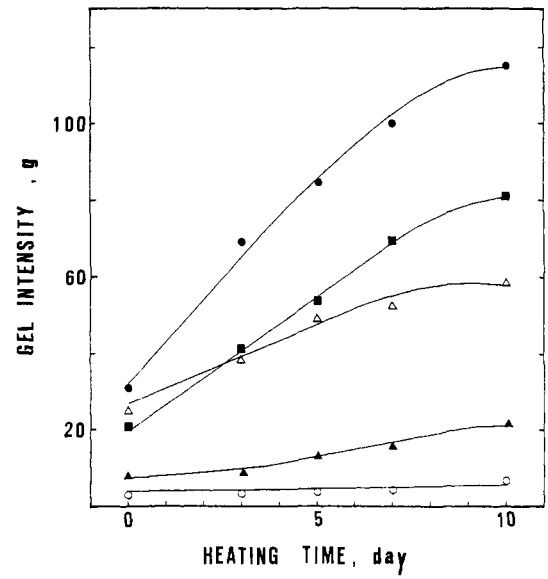

Figure 2. Effect of some structure-modifying agents on the gel strength of heated DEW in the dry state for various periods of time (10% protein concentration): ●, water; ■, 20 mM 2-ME; △, 3 M urea; ▲, 6 M urea; ○, 1% SDS.

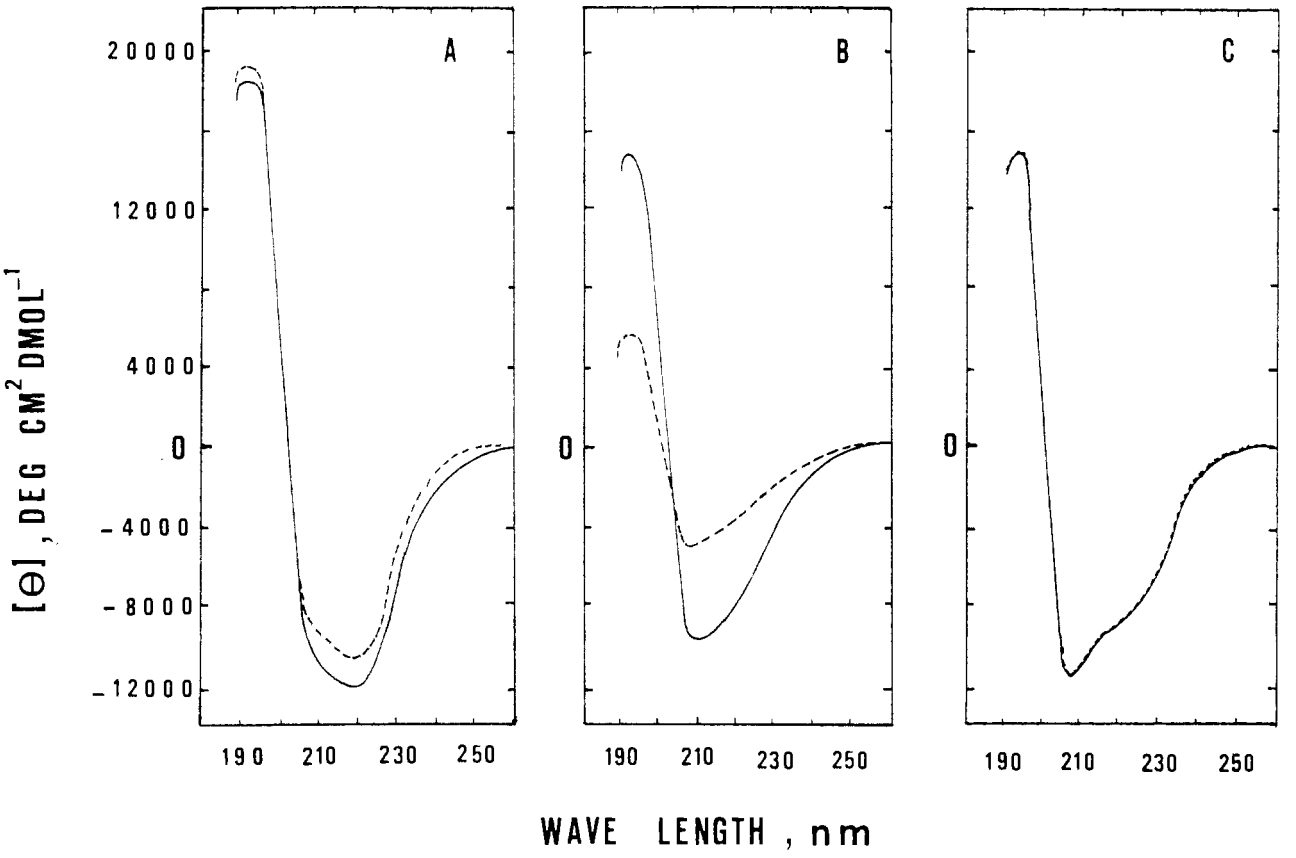

Figure 3. Effect of heating in the dry state on the CD spectra of ovalbumin (A), ovotransferrin (B), and lysozyme (C): (—) nonheated sample; (- - -) sample heated for 5 days.

To investigate the forces involved in formation of the firm gel from DEW upon heating in the dry state, the thermal gelation of DEW heated in the dry state for various periods was measured in the presence of protein structure modifying reagents that are known to destabilize specifically intermolecular reactive forces. The data are shown in Figure 2. It was observed that 2-mercaptoethanol decreased the gel strength of DEW to some extent at any heating time. The effect was almost the same for unheated and heated samples in the dry state, indicating the partial involvement of sulfhydryl–disulfide interchange reaction in the thermal gelation of heated DEW in the dry state. On the other hand, urea dramatically lowered the gel strength of heated DEW in the dry state depending on the concentration. Urea is known to destabilize both hydrogen-bonding and hydrophobic interactions. In addition, the gels formed in the presence of SDS were very weak at any heating time. This suggests the inhibition of protein–protein interactions due to hydrophobic forces by the binding of this anionic detergent to protein molecules. These results, therefore, suggest that the strengthening of gel formed from DEW heated in the dry state is mainly attributed to hydrogen-bonding and hydrophobic interactions and partially to the formation of intermolecular disulfide bonds. Evidence for the role of hydrogen bonds in gels has been given for insulin (Burke and Rougvie, 1972) and bovine serum albumin (Yasuda et al., 1986). In both cases, the gel network might depend on the formation of intermolecular β-sheets. Kato and Takagi (1988) have reported that intermolecular β-sheet structure increases in heat-induced soluble aggregates of ovalbumin in proportion to increases in their molecular size. Accordingly, it is possible that exposure of large numbers of hydrophobic residues to the surface of egg white proteins heated in the dry state (Kato et al., 1989) might enhance the formation of intermolecular hydrogen bonds through β-sheet conformation and thus lead to the formation of a firm and stable gel network.

Table I. Rate of Increase in the Digestion Velocity of Ovalbumin and Ovotransferrin as a Function of Heating Time in the Dry State

	relative velocity[a]	
heating time, days	ovalbumin	ovotransferrin
0	1.00	1.00
3	1.15	1.35
5	1.60	2.16

[a] Digestion velocity was estimated as described in Materials and Methods. These values of heat-treated samples represent the ratio to nonheated samples (0 day).

CD spectra of ovalbumin, ovotransferrin, and lysozyme heated at 80 °C for 5 days in the dry state are shown in Figure 3. The CD spectra of ovalbumin indicated only slightly conformational changes by heating in the dry state for 5 days (Figure 3A). CD spectra of lysozyme indicated no change upon heating for 5 days in the dry state, as shown in Figure 3C. On the other hand, when ovotransferrin was heated at 80 °C for 5 days in the dry state, there was a marked change in the CD curve (Figure 3B), indicating a considerable conformation change. The differences in the CD spectra seem to result from differences in denaturation behavior of the three proteins, because denaturation temperatures of lysozyme, ovalbumin, and ovotransferrin are 81, 77, and 60 °C, respectively. Attempts to verify the occurrence of refolding after dry-heating were carried out in both protein solution and dry powdered protein. There were no detectable differences in CD spectra between freshly prepared solutions and stored solutions for 1 day at room temperature of

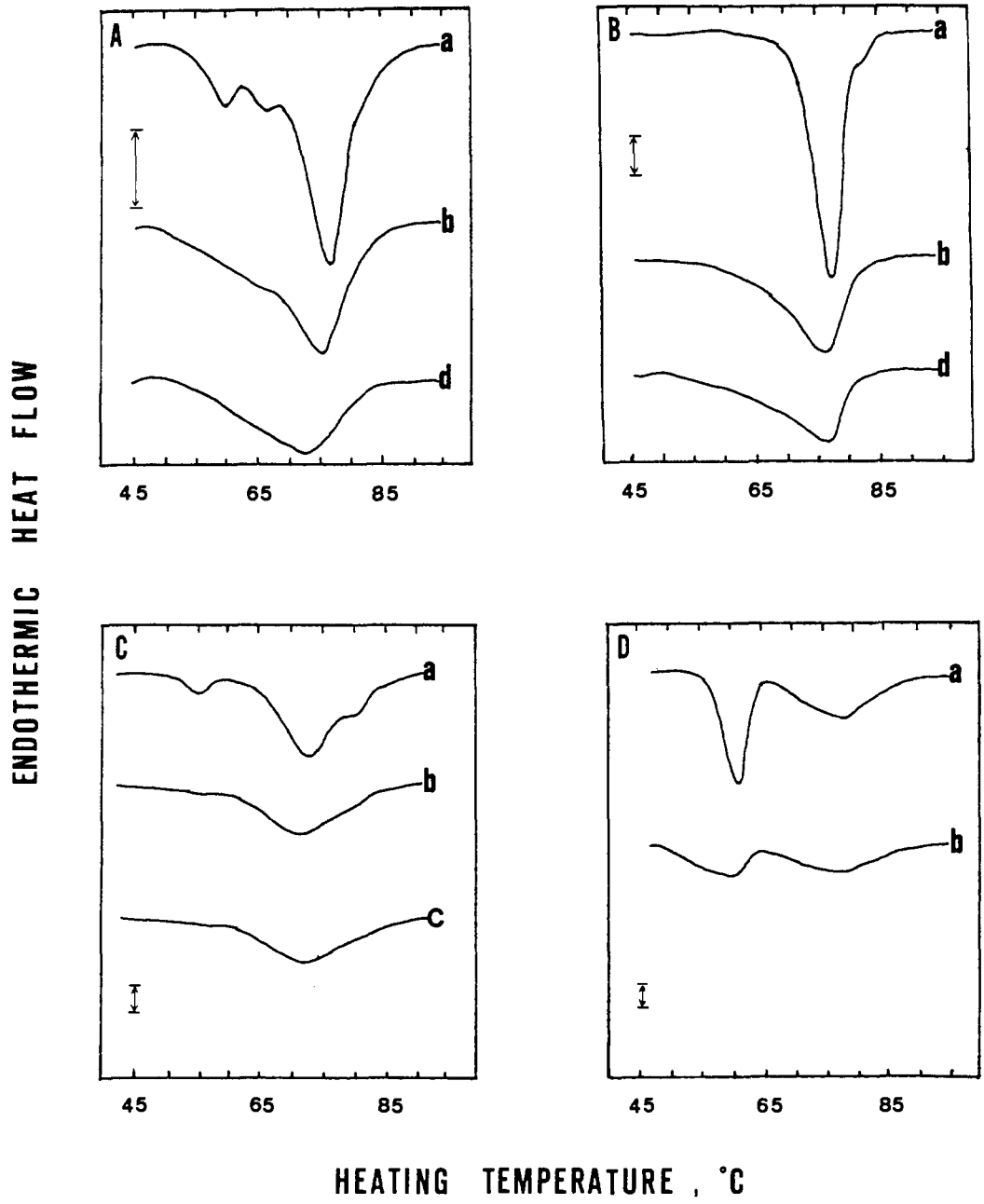

Figure 4. DSC thermograms of DEW (A), ovalbumin (B), globulin fraction (C), and ovotransferrin (D). Samples were heated in the dry state for different lengths of time. Key: (a) nonheated; (b) heated for 5 days; (c) heated for 7 days; (d) heated for 10 days. Vertical arrow represents 0.3 mW.

proteins dry-heated for various lengths of time. In addition, no differences in CD spectra were observed between freshly dry-heated proteins and those stored in the dry state at room temperature for 1 month. These suggest the irreversibility of dry-heating denaturation. Similarly, the gel strengths of DEWs heated in the dry state for various periods were completely consistent with those of fresh ones and those stored in the dry state at room temperature for 1 month. These data strongly indicate a favorable advantage of such heat treatment, particularly for food processing.

Susceptibilities to chymotrypsin of ovalbumin and ovotransferrin heated in the dry state for various periods of time are shown in Table I. For a better illustration of the data, Table I represents the rate of increase in the chymotryptic digestion velocity, calculated from V_h/V_n, where V_h is the digestion velocity of heated sample and V_n is the digestion velocity of native sample. Susceptibility to proteolysis gradually increased with an increase of heating time in the dry state. A greater increase in proteolysis was observed for heated ovotransferrin compared to heated ovalbumin. The rate of protein digestibility by proteolytic enzyme depends on the conformation of proteins (Green and Neurath, 1954). Changes in the conformation can be detected more sensitively by protease digestibility than by routine optical measurements such as CD and differential spectrum (Imoto et al., 1976; Ueno and Harrington, 1984; Kato et al., 1985). Imoto et al. (1976) and Kato et al. (1985) have proposed that susceptibility to proteases is proportional to the flexibility of protein structure. Thus, the increase of protease digestibility of proteins heated in the dry state indicates that conformations were altered toward more flexible conformations. Although heating at 80 °C in the dry state for 5 days induced little change in the conformation of ovalbumin as shown in the CD spectra (Figure 3A), a considerable increase in susceptibility to proteolysis was observed. A significant increase in the susceptibility to

Table II. Thermal Characteristics and Gel Strength of Egg White Proteins under Various Heating Times in the Dry State[a]

heating time, days	T_d, °C peak 1	peak 2	peak 3	peak 4	peak 5	ΔH, J/g	gel strength, g
			DEW				
0	60.2	66.7	–	77.0	–	12.6	29.3
5	d	d	–	75.4	–	9.1	84.7
10	d	d	–	73.4	–	5.9	115.0
			Globulin Fraction				
0	60.8	–	74.8	–	80.9	11.9	72.3
5	d	–	73.7	–	d	8.1	93.8
7	d	–	73.3	–	d	7.9	114.8
			Ovalbumin Fraction				
0	61.3	–	–	77.3	82.3	15.7	21.4
5	60.6	–	–	76.7	81.9	13.6	50.9
7	d	–	–	76.5	81.7	11.6	63.6
			Purified Ovalbumin				
0	–	–	–	77.7	–	13.6	3.7
5	–	–	–	76.3	–	10.5	18.2
10	–	–	–	76.1	–	8.9	35.0
			Ovotransferrin				
0	61.3	–	74.6	–	–	12.8	15.6
5	60.6	–	73.7	–	–	7.7	6.8

[a] Key: d, disappearance of the peak; –, peak not detectable.

proteolysis was observed for ovotransferrin heated in the dry state for 5 days, reflecting extensive conformational changes as predicted from CD spectrum. This may suggest the positive correlation between gel formation and protein flexibility that is enhanced by heating in the dry state.

The thermal characteristics of DEW, purified ovalbumin, crude globulin and crude ovalbumin fractions prepared by half-saturated ammonium sulfate precipitation, and ovotransferrin as a function of heating time in the dry state are shown in Figure 4 and Table II. When 10% protein samples were analyzed, DEW (Figure 4A) exhibited three endothermic peaks with a total enthalpy of 12.6 J/g. Purified ovalbumin (Figure 4B) showed a fairly sharp peak with a T_d value of 77.7 °C and an enthalpy of 13.6 J/g. The thermogram of the globulin fraction (Figure 4C) shows three endothermic peaks with a total enthalpy of 11.9 J/g. Ovotransferrin, containing a considerable amount of impurities (Figure 4D), shows a major peak with T_d 61.3 °C and enthalpy 12.8 J/g. When the samples were heated in the dry state for 5 days, there was a broadening of the peaks with a detectable decrease in T_d values and marked decrease in ΔH. A further increase in heating time in the dry state resulted in an additional decrease of both T_d and ΔH and more broadening in the endothermic peaks. Wright et al. (1977) demonstrated that the sharpness of an endothermic peak is indicative of cooperativity of the transition from the native to the denatured state. Also, it has been well documented that broadening of peaks indicates the existence of intermediate forms different from the native form. In other word, the conformation of the protein molecule has shifted toward the unfolded state. Thus, partially unfolded conformations formed by preheating prior to thermal gelation may play an important role in the gelation process, probably by promoting more extensive intermolecular interaction. The results in Table II and Figure 4 show that when egg white proteins were heated in the dry state, there was a marked decrease in the enthalpy with a slight decrease in T_d, accompanied by a significant increase in the gel strength at all cases, except ovotransferrin. Ovotransferrin was too sensitive to dry-heating in terms of gelation; it coagulated easily even by heating in solution. Therefore, it seems likely that extensive protein

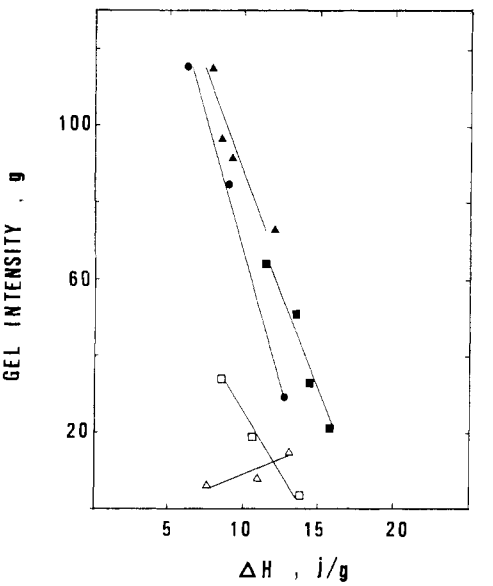

Figure 5. Gel strength versus ΔH plots for egg white proteins: (●) DEW; (▲) globulin fraction; (■) ovalbumin fraction; (□) purified ovalbumin; (△) ovotransferrin. ΔH were calculated from the entire thermogram of protein samples.

structural changes are not required for gelation. The relationships between ΔH and gel strength of DEW and some proteins heated in the dry state for various days are shown in Figure 5. A good correlation was observed between the decrease in ΔH and the increase in gel strength of DEW. This result shows that the decrease in enthalpy may result in extensive unfolding and play an important role in the thermal gelation process, probably by promoting moreextensive protein interaction for gelation. Similar relationships were investigated between ΔH and the gel strength of crude ovalbumin and globulin fractions, which were separated only by ammonium sulfate precipitation. Good correlation between ΔH and gel strength was obtained in crude ovalbumin and globulin fractions, while the plots of purified ovalbumin and crude ovotransferrin fractions deviated significantly from the regression line. Firm gels were obtained fromthe globulin and ovalbumin fractions including considerable amounts

Table III. Gibbs Free Energy of Unfolding (ΔG) of DEW and Ovalbumin upon Heating for 10 Days in the Dry State

heating time, days	ΔG,[a] J/mol	
	DEW	ovalbumin
0	13.39	20.92
10	6.28	14.64

[a] Calculated as described in Materials and Methods.

of other proteins. The weak gels were obtained from purified ovalbumin, while gel strength of ovotransferrin was scarcely correlated with ΔH values. This suggests that heterogeneous interaction between several proteins may be important for the formation of a firm gel. It is interesting that the gel strength of the globulin fraction is much stronger than that of the ovalbumin fraction. This may be because ovalbumin is more stable to heat treatment than the globulin fraction. The value of ΔH obtained from the thermograms of DSC generally reflects competition between endothermic unfolding and exothermic aggregation. Therefore, the effects of exothermic aggregation on ΔH may also be partially involved, but the lower T_d values and enhancement of flexibility described above confirm the effect of endothermic unfolding on ΔH.

To examine the interference of exothermic aggregation on the values of ΔH obtained from DSC analysis, the Gibbs free energies of unfolding in water, ΔG, of DEW and ovalbumin were determined (Table III). Big changes in ΔG were observed between native and dry-heated proteins. The rate of decrease in ΔG was 53% in DEW and 30% in ovalbumin by dry-heating for 10 days. The similar rate of decrease in ΔH was observed in DEW and ovalbumin by dry-heating for 10 days (Table II). This provides direct evidence that exothermic aggregation during DSC analysis had no significant effect on the ΔH values. Therefore, the strong correlation between the increase in gel strength and the decrease in ΔH values (Figure 5) suggests the major contribution to the significant increase of gel-forming ability to be the endothermic unfolding of egg white proteins by heating in the dry state.

In conclusion, the present study clearly indicates that heating egg white proteins at 80 °C in the dry state is a critical step in the enhancement of protein flexibility and the reduction of protein stability. This might be reflected by the significantly increased functionality (foaming, emulsifying, and gelling properties), as described in our previous paper (Kato et al., 1989). A good correlation between gel strength and ΔH was obtained for dried egg white proteins used in this study. On the basis of this study, the general mechanism of thermal gelation of egg white proteins heated in the dry state can be demonstrated. The extent of gel network formation of proteins depends on the relative magnitude of ΔH and protein species. Proteins whose ΔH lowered by preheating in the dry state are liable to denature faster than native proteins possessing higher ΔH during heat for gelation. Thus, dry-heating of DEW would facilitate the exposure of a greater number of functional groups available for cross-link formation to stabilize the gel network. In addition, the greater extent of protein flexibility of proteins heated in the dry state might facilitate the formation of an ordered three-dimensional network of gel. It can also be concluded that the forces involved in the formation of the significant gel network from egg white proteins heated in the dry state are proven to be mainly the enhancement of the intermolecular hydrogen-bonding and hydrophobic interactions, including the additive sulfhydryl–disulfide interchange reaction. Since linear correlation was obtained between increased gel strength and the decreased ΔH of dry-heated proteins, the optimum gel of DEW may easily be obtained by controlled heating in the dry state for a certain time. In parallel, the effectiveness of dry-heating may enhance the use of egg white proteins in food and pharmacological applications requiring high gelation, emulsification, or foaming properties.

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