Thermally Induced Changes in Egg White Proteins

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The heat-induced aggregation of egg white protein and conformational change of heat-denatured egg white protein at a high pH value were investigated. The flexibility and surface hydrophobicity of egg white protein increased with increasing temperature. The results of the changes in sulfhydryl groups in egg white protein during heating and SDS-PAGE indicated that sulfhydryl-disulfide interchange between the heat-denatured egg white protein had occurred. The change in CD spectra of heat-denatured egg white protein was interpreted as the result of increase in β-sheet structure in sacrifice of helical structure. The partially unfolded stable structure of heat-denatured egg white protein was predicted from the cooperativity of the guanidine hydrochloride denaturation curves. It was concluded from these results that heat-induced soluble egg white aggregates were built from the partially unfolded molecules which contain a considerable amount of secondary structure and were cross-linked with a continuous disulfide cross-linked network and that the network of the β-sheet structure was strengthened by the exposed hydrophobic residues.

INTRODUCTION

Egg white exhibits characteristics critical to the preparation of many food products such as water-binding properties, excellent foaming and emulsification capacities, high nutritive values, and the ability to coagulate and form gels upon heating. Heat coagulation is one of the important functional properties of egg white. Though the coagulation of egg white protein has been extensively studied by many investigators (Nakamura et al., 1978; Hegg and Lofgvist, 1979; Shimada and Matsuskita, 1980; Egelandsdal, 1980; Ma and Holme, 1982; Watanabe et al., 1985; Kato et al., 1986; Matsudomi et al., 1986, 1987), the thermal denaturation of egg white protein is least understood. It is important to make clear what kind of interactions are responsible for the heat-induced aggregation among heterogeneous proteins in egg white. Since egg white forms soluble aggregates when it is aqueous solution at alkaline pH and low ionic strength, it is convenient to study the conformational changes that accompany the thermal denaturation and aggregation process. In this paper, we report the factors contributing to the heat-induced aggregations of egg white proteins and conformational changes of heat-denatured egg white proteins to elucidate the mechanism of aggregation.

MATERIALS AND METHODS

Egg white from which the chalaza was removed was stirred gently with a magnetic stirrer so as not to produce foam for 1 h at room temperature, giving homogeneous egg white. The egg white was diluted with an equal volume of water and dialyzed against distilled water. The precipitates formed were removed by centrifugation.

Heat treatment of egg white protein was done as follows. Five milliliters of 2.0% egg white protein solution in distilled water, pH 9.5, was put in a test tube that was tightly closed with screw caps to prevent evaporation of water during heating and then heated in an incubator at the rate of 2 °C/min from 20 °C to a given temperature. As soon as the sample was heated to a given temperature, the test tube was taken from the incubator and cooled at room temperature by ice water and subsequently used in experiments as follows.

Measurement of flexibility of heat-denatured egg white protein was carried out by the protease digestion method. To 4 mL of 1.0% protein solution in 0.05 M Tris-HCl buffer, pH 8.0, was added 250 μL of 1.0% α-chymotrypsin (Novo Industry) solution. The enzymatic reaction was carried out at 38 °C for 15 min. After protease digestion, 4 mL of 4% aqueous trichloroacetic acid was added, and then the precipitate was removed by filtration with filter paper (Toyo Roshi Ltd., No. 2). The amount of digests in the filtrate was estimated by the Lowry method (Lowry et al., 1951). The extent of digestion was expressed as the percentage of total protein digested.

Measurement of surface hydrophobicity of protein in heat-denatured egg white was carried out by the method of Kato and Nakai (1980) using a fluorescence probe, *cis*-parinaric acid. Ten microliters of an ethanolic solution of *cis*-parinaric acid was added to 2 mL of 0.1% protein solution in 0.01 M phosphate buffer, pH 7.4. The mixture was excited at 325 nm, and the relative fluorescence intensity was measured at 420 nm in a fluorescence spectrophotometer (Hitachi Model 204). The relative fluorescence intensity reading was adjusted to 1.0 when 10 μL of *cis*-parinaric acid solution was added to 2 mL of 0.01 M phosphate buffer, pH 7.4, in the absence of protein. The initial slope (S_0) was calculated from the fluorescence intensity vs protein concentration plot.

Measurement of sulfhydyl groups was performed by using Ellman's reagent (Beveridge et al., 1974). To 1 mL of the sample solution was added 1 mL of 0.1 M Tris-glycine buffer, pH 8.0, containing 0.01 M EDTA with or without 5% SDS and then 0.05 mL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution (40 mg of DTNB in 10 mL of 0.1 M Tris-glycine buffer at pH 8.0). The color absorbance was read at 412 nm.

SDS slab polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970), using 10% acrylamide separating gel and 4.5% stacking gel containing 0.1% SDS. Protein samples were prepared in 0.125 M Tris-HCl buffer, pH 6.8, containing 2% SDS and 20% glycerol with or without 0.5% 2-mercaptoethanol. Electrophoresis was carried out at a constant current of 30 mA for 2 h with an electrophoresis buffer of 0.125 M Tris-glycine, pH 8.6, containing 0.1% SDS. Then the gel was stained with Coomassie brilliant blue and destained in methanol/acetic acid/water (20:10:70 v/v/v).

Circular dichroism (CD) measurements were carried out with a Jasco J-500 spectropolarimeter equipped with a data processor (Model DP-50). CD spectra were measured at protein concentration of 0.5 mg/mL using a cell with a 0.10-cm light path in the wavelength range 200-260 nm. CD spectra were expressed in terms of mean residue ellipticity (deg cm^2 $dmol^{-1}$). The accuracy of the data was improved by averaging eight scans integrated with the data processor. CD curves thus obtained were simulated by a nonlinear least-squares curve-fitting program that was written essentially according to the method of Chiang et al. (1978). The estimation of α-helix, β-sheet structure, β-turn, and unordered form was carried out in the computed curves giving the best fit.

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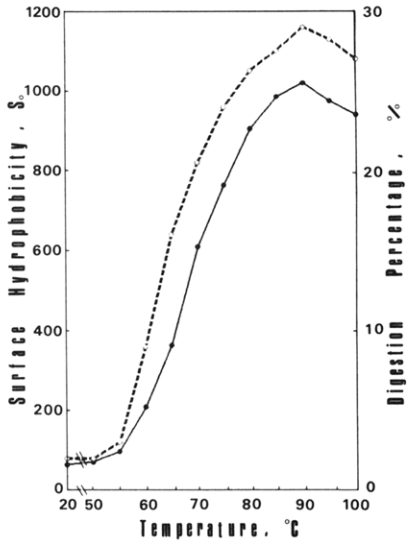

Figure 1. Thermal denaturation curves obtained by monitoring the surface hydrophobicity and proteolytic digestion of egg white protein. (●) Surface hydrophobicity; (○) proteolytic digestion.

The effect of guanidine hydrochloride on the secondary structure of heat-denatured egg white was studied as follows: 1–5 M guanidine hydrochloride was added to a heat-denatured egg white solution cooled to 25 °C after heating at 100 °C, and then the CD spectrum was measured.

RESULTS

Thermal denaturation curves obtained by monitoring the surface hydrophobicity and flexibility of egg white protein are shown in Figure 1. No coagulation occurred during heating of proteins. The surface hydrophobicity of protein is known to change in proportion to the conformational change detected by routine optical methods, such as circular dichroism (Kato et al., 1981). The surface hydrophobicity and digestion velocity increased as the result of heating over the temperature range 60–90 °C. The transition points determined from the curves for surface hydrophobicity and digestion velocity of egg white were 55 and 50 °C, respectively.

The average number of total available sulfhydryl (SH) groups and disulfide (SS) bonds found in native egg white protein were 50.0–51.7 μmol/g of native egg white protein (Feeney et al., 1966) and 79.9–84.5 μmol/g of native egg white protein (Beveridge and Arnfield, 1979), respectively. The change in sulfhydryl groups was measured with DTNB in the absence (surface sulfhydryl groups) or in the presence of 2.5% SDS (total sulfhydryl groups). Figure 2 shows the changes in sulfhydryl groups in egg white protein during heating. The surface sulfhydryl groups in egg white protein showed significant changes up to 70 °C but started to decrease at a heating temperature above 80 °C. On the other hand, the amount of total sulfhydryl groups of egg white protein decreased gradually at a heating temperature above 70 °C. These results indicate that most sulfhydryl residues in egg white protein existed in the interior of protein molecules and were exposed with heat denaturation and formed S–S bonds by SH oxidation or SH/S–S interchange reaction.

The SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to assess the binding type of heat-induced aggregation of egg white protein. From the SDS–PAGE patterns in the presence of 2-mercaptoethanol (Figure 3A), there were no changes in the bands at any heating temperature employed here, indicating that the soluble aggregate was almost dissociated by SDS and

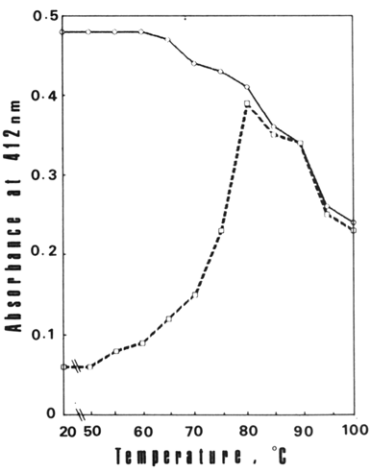

Figure 2. Changes of sulfhydryl groups in egg white protein during heating. (□) Surface SH groups; (○) total SH groups.

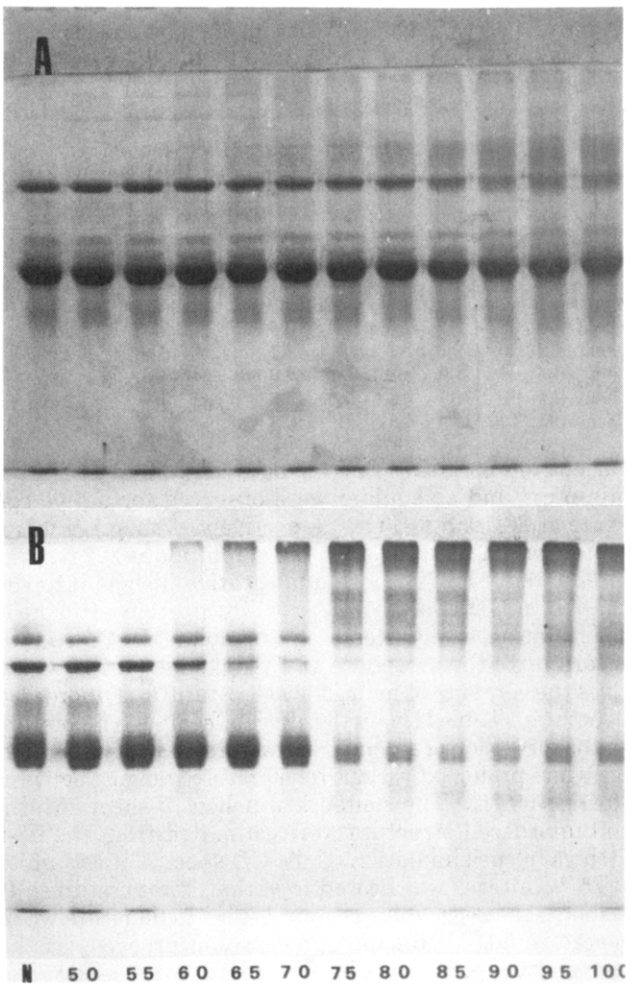

Figure 3. SDS–polyacrylamide gel electrophoresis patterns in the presence (A) or in the absence (B) of 2-mercaptoethanol.

2-mercaptoethanol. But when SDS–PAGE was performed in the absence of 2-mercaptoethanol (Figure 3B), the bands' lower mobility was increased at a heating temperature above 60 °C. Most of the aggregates remained undissociated. In light of this fact, the main type of binding of soluble aggregate is likely to be disulfide bonds between egg white proteins. These results suggest that sulfhydryl–disulfide interchange reactions occurred during heating.

The effects of heating on the structure of egg white were studied by CD analysis. Figure 4 shows typical CD spectra of nonheated and heat-denatured egg white protein at

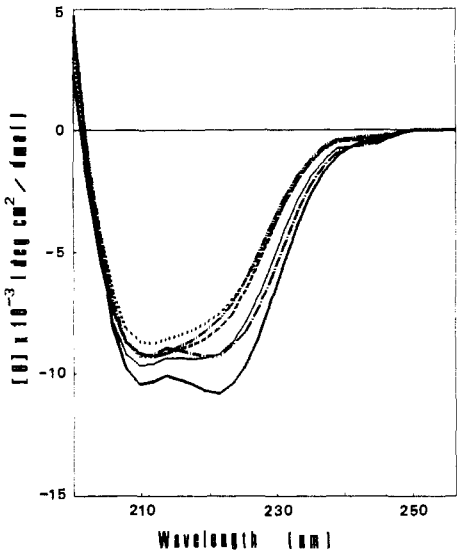

Figure 4. Circular dichroism spectra of native and denatured egg white protein. (—) Native egg white proteins; egg white proteins heated at 60 (- - -), 70 (—), 80 (- - - -), 90 (- - - -), and 100 °C (- - - - - -), respectively.

Table I. Changes in the Contents of the Secondary Structure of Egg White Proteins during Heat Denaturation

egg white proteins	fractions of secondary structure			
	helix, %	β-sheet, %	β-turn, %	unordered, %
native	40.6	15.8	15.5	28.2
heated at 60 °C	36.9	16.5	17.9	28.6
heated at 70 °C	27.9	29.1	13.6	29.3
heated at 80 °C	23.3	37.6	8.6	30.5
heated at 90 °C	19.5	43.5	6.0	31.0
heated at 100 °C	15.8	46.8	5.5	31.9

various temperatures ranging between 60 and 100 °C. A minimum and a shoulder were observed for native egg white at 222 and 210 nm, respectively. When egg white protein was heated to 60° C, the amplitude at 222 nm was attenuated. To get more quantitative insight into the change in CD spectra of egg white protein after heat denaturation, it was attempted to simulate the CD spectra according to the procedure of Chiang et al. (1978), which gives the contents of helix, β-sheet, β-turn, and unordered structures. Table I shows the effect of heating temperature on the formation of secondary structures of heat-denatured egg white protein at various temperatures ranging between 60 and 100 °C. The contents of helical, β-sheet, β-turn, and unordered structure were estimated from the best fittings in the simulation of the CD spectra of egg white at 25 °C after it was heated to various temperatures. It is shown that egg white protein begins to denature when heated to 60 °C and the denaturation proceeds as the temperature increases. The helix content decreased to less than half, while the β-sheet content increased more than 3-fold. The β-turn content of egg white protein also decreased with heat denaturation. Thus, a marked increase of β-sheet structure in sacrifice of helical structure was observed during heat denaturation of egg white protein.

Figure 5 shows the guanidine hydrochloride denaturation curves of egg white before and after heat treatment at 100 °C. The extent of denaturation was determined by measuring the ellipticity at 222 nm, since the negative CD value at 222 nm is characteristic of ordered structure. The ellipticity of native egg white protein did not change up to 2 M guanidine hydrochloride, while that of denatured egg white protein gradually lowered even in the range 0–5 M guanidine hydrochloride. The guanidine hydrochloride

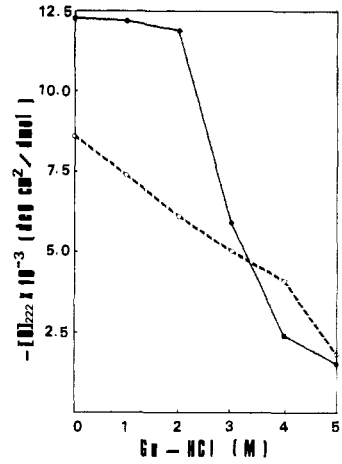

Figure 5. Guanidine hydrochloride denaturation curves of native and heat-denatured egg white protein. (●) Native egg white proteins; (○) egg white proteins heated at 100 °C.

denaturation curves of heat-treated egg white protein also indicated that alternative regular structure was formed by the heat denaturation.

DISCUSSION

Each protein species in the egg white protein forms a soluble aggregation on heating used in this study (pH 9.5 and low ionic strength). At pH regions far from the isoelectric points of the main protein components of egg white, mutual repulsion of protein molecules increased, so that clumps of coagulum were not formed.

The exposed hydrophobic residues on the molecular surface remarkably increased with the heat denaturation of egg white proteins, and the formation of disulfide bonds probably involved in the first step of aggregation and heating causes egg white proteins to polymerize by intermolecular sulfhydryl–disulfide exchange (Figures 2 and 3).

CD spectra of heat-treated egg white protein were measured to examine changes in the secondary structure of egg white proteins. The change in CD spectra during heat denaturation was interpreted as a result of an increase in β-sheet structure at the sacrifice of helical structure. The "molten" structure that is partially unfolded and more flexible than the native form may be formed by heat denaturation. It is interesting that the guanidine hydrochloride denaturation curves of heat-treated egg white protein indicate the cooperativity, although it is lower than that of native egg white protein. This suggests that the partially unfolded stable structure is formed by the heat treatment of egg white proteins. These results are in good accordance with the results of Kato and Takagi (1988) that intermolecular β-sheet structure is formed during heat denaturation of ovalbumin using the curve-fitting analysis of CD spectra.

Recently, Van Kleef (1986) reported that ovalbumin, the major protein of egg white, unfolds completely when heated at a high pH value (pH 10) solution. The unfolded, randomly coiled ovalbumin molecules are mainly cross-linked via covalent disulfide cross-links. We have proposed that soluble egg white aggregates are built from the partially unfolded molecules which contain a considerable amount of secondary structure and are cross-linked with a continuous disulfide cross-linked network and that the network of the β-sheet structure is strengthened by the exposed hydrophobic residues.

Egg white comprises heterogeneous proteins such as ovalbumin, conalbumin, ovomucoid, and lysozyme. The heat

aggregation of egg white could be facilitated by interactions among such heterogeneous proteins. But in this paper, we did not discuss in detail the interactions among these proteins. Further studies to elucidate the mechanism of the aggregate formation among the heterogeneous proteins in egg white are now in progress.

ACKNOWLEDGMENT

We are much indebted to Dr. H. Tsuchiya, Associate Professor of Sophia University, for measuring CD spectra.

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Received for review January 8, 1990. Accepted June 20, 1990.