Heat-Induced Changes in the Secondary Structure of Hen Egg S-Ovalbumin

Thomas J. Herald† and Denise M. Smith*

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824-1224

Solutions of hen egg S-ovalbumin at pD or pH 3.0, 7.0, and 9.0 were heated at temperatures between 30 and 90 °C to study changes in secondary structure by Fourier transform infrared spectroscopy (FTIR), storage moduli by dynamic rheological testing, and denaturation temperatures by differential scanning calorimetry. Second-derivative infrared spectra of native S-ovalbumin at pD 7.0 and 9.0 revealed protein absorption bands for β-sheet (1626 cm^{-1}), 3_{10}-helix (1638 cm^{-1}), α-helix (1656 cm^{-1}), and turns (1682 and 1670 cm^{-1}). The β-sheet absorption band decreased to a shoulder for ovalbumin at pD 3.0. Bands at 1626, 1638, and 1656 cm^{-1} decreased as heating temperature was increased, whereas bands at 1614 and 1682.5 cm^{-1}, representing hydrogen-bonded β-strands, increased in intensity. Changes in secondary structure were closely correlated to denaturation temperatures and increases in storage moduli of S-ovalbumin solutions. Results help elucidate texture formation in egg products by establishing a relationship between changes in protein secondary structure and viscoelasticity with heat.

INTRODUCTION

Ovalbumin is the major hen egg white protein that contributes gel properties to egg-containing food systems. Heat-induced gelation is a two-step process involving protein denaturation and aggregation to form a cross-linked matrix (Ferry, 1948). Monitoring heat-induced changes in the secondary structure of ovalbumin will help to further the understanding of the mechanisms involved in gelation and texture formation by egg proteins.

The crystal structure of ovalbumin was reported recently (Stein et al., 1991). The secondary structure of native ovalbumin and S-ovalbumin has been determined using Raman spectroscopy and circular dichroism. Painter and Koenig (1976) did not detect any conformational difference between ovalbumin and S-ovalbumin in 0.05 M NaCl by Raman spectroscopy. Both proteins were reported to contain 50% random coil, 25% α-helix, and 25% β-sheet. Kint and Tominatsu (1979) reported ovalbumin solutions in 0.05 M KCl, pH 8.0, contained a higher α-helix content than β-sheet. The same authors reported S-ovalbumin had a 3–4% higher β-sheet content than ovalbumin.

Using circular dichroism (CD), Egelandsdal (1986) reported that ovalbumin in solution at pH 9.5 contained 30% helix and 40% β-sheet. The helical content increased when ovalbumin was solubilized in 0.04 M NaCl as compared to water. Doi et al. (1987) using CD observed 49% helix, 13% β-sheet, and 24% unordered structure in native ovalbumin. Several authors have observed an increase in β-sheet and a loss of helix by CD when ovalbumin was heated (Doi et al., 1987; Kato and Takagi, 1988). Circular dichroism cannot identify turns or distinguish between 3_{10}- and α-helices (Susi and Byler, 1988; Prestrelski et al., 1991).

The secondary structure of proteins can also be examined using Fourier transform infrared spectroscopy (FTIR) with second-derivative analysis. The amide I band (1620–1700 cm^{-1}), caused by carbonyl stretching vibration of the peptide backbone, is used to monitor secondary structure of proteins by FTIR (Byler and Susi, 1986; Surewicz and Mantsch, 1988). Vibrational transitions associated with α-helix, 3_{10}-helix, β-sheet, turns, and unordered structure produce bands at specific frequencies in the amide I region (Prestrelski et al., 1991). Second-derivative spectra analysis improves resolution of overlapping bands in FTIR spectra, providing a qualitative means for following subtle change in protein conformation (Byler and Susi, 1988; Byler and Purcell, 1989).

There is better correlation between FTIR and X-ray measurements of protein secondary structure than between X-ray data and CD or Raman spectroscopy (Susi and Byler, 1988). Fourier transform infrared spectroscopy has been used to investigate secondary structure of whey proteins (Byler and Purcell, 1989; Prestrelski et al., 1991), bovine serum albumin, carbonic anhydrase, lysozyme (Byler and Susi, 1986), hemoglobin, and ribonuclease A (Susi and Byler, 1983). The effects of heat on the secondary structure of proteins have been investigated using β-lactoglobulin, α-lactalbumin, bovine serum albumin (Byler and Purcell, 1989; Clark et al., 1981), and soy 11S globulin (Chen et al., 1990).

No research has been reported using FTIR to resolve the secondary structure of ovalbumin and subsequent conformational changes due to heating and pH. The objectives of the present research were to monitor changes in secondary structure of S-ovalbumin by FTIR as a function of temperature at pD 3.0, 7.0, and 9.0. Changes in the secondary structure of ovalbumin with temperature were compared to denaturation transition temperatures determined by differential scanning calorimetry (DSC) and gel formation as measured by dynamic rheological testing.

MATERIALS AND METHODS

Materials. Ovalbumin (grade V, lot 19F 8105) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Purity was confirmed by the presence of a single protein band on sodium dodecyl sulfate–polyacrylamide electrophoresis (Laemmli, 1970). Ovalbumin was in the S form as determined by differential scanning calorimetry (Donovan and Mapes, 1976). Deuterated reagents were purchased from Merck and Co. (St. Louis, MO).

Fourier Transform Infrared Spectroscopy. S-Ovalbumin and all reagents were stored in a vacuum desiccator with

† Present address: Department of Foods and Nutrition, Kansas State University, Manhattan, KS 66506.

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phosphorous pentoxide. Solutions were prepared in a glovebox under an atmosphere of nitrogen, dried by passing through $CaSO_4$ desiccant prior to entering the glovebox.

S-Ovalbumin was prepared as 1.75% (w/v) solutions in 25 mM KD_2PO_4 in D_2O. The pD of the *S*-ovalbumin solution was adjusted with 30% DCl or 40% NaOD to pD 3.0, 7.0, or 9.0 by adding 0.4 to the pH reading (Covington et al., 1968) measured using a Corning pH meter (Model 107, Corning, NY). *S*-Ovalbumin solutions (200 μL) were placed in capped glass vials (2-mL capacity) which were purged with dry nitrogen for 2 min before heating at a constant temperature in a Fisher Programmable Isotemp oven (Model 230F, Pittsburgh, PA) for 15 min. *S*-Ovalbumin at pD 7.0 and 9.0 was heated at 80, 85, and 90 °C. Heat treatments at pH 3.0 were 60, 65, 70, and 80 °C. After heating, samples were placed inside the glovebox and allowed to stand for approximately 5 min until cool. Cooled samples were purged with dry nitrogen gas for 2 min, and 100 μL was transferred to a circular demountable cell (Model P-3 N930-1117, Perkin-Elmer, Norwalk, CT) with CaF_2 windows and Teflon spacers (75-μm path length), which had been purged with dry nitrogen gas for 10 min before sample loading. Infrared spectra were collected at ambient temperature using a Model 1800 FTIR spectrometer (Perkin-Elmer) equipped with an incandescent wire source, a potassium bromide coated beam splitter, and a broad-range mercury/cadmium/telluride detector. Before the IR spectrum was recorded, the sample chamber and cell were purged with dry nitrogen gas for 15 min. All spectra were scanned 1000 times and recorded at a resolution of 2 cm^{-1}. The sample chamber was continuously purged with dry nitrogen. Resolution enhancement by second-derivative analysis (CDS-3 applications software, Perkin-Elmer) was performed using the Savitzky–Golay derivative routine (Savitzky and Golay, 1964) and a 13-data point (13 cm^{-1}) window. Spectral contributions from a D_2O blank and residual water vapor were subtracted. Each treatment was tested in triplicate. The secondary structure of ovalbumin was determined on the basis of FTIR spectra of proteins with known secondary structures (Clark et al., 1981; Byler and Susi, 1986; Susi and Byler, 1988; Halloway and Mantsch, 1989; Levitt and Greer, 1977; Dong et al., 1990; Acharya et al., 1989).

Differential Scanning Calorimetry. A Du Pont 990 thermoanalyzer (Wilmington, DE) equipped with a Du Pont 910 cell base and standard DSC cell were used to determine onset denaturation temperature (T_o) and thermal denaturation temperature (T_d) of ovalbumin (80 mg/mL) at pH 3.0, 7.0, and 9.0 in 25 mm KH_2PO_4. Approximately 15 mg of accurately weighed ovalbumin solution was sealed in a Du Pont aluminum hermetic pan (900793-901) and lid (900794-903). Samples were heated from 30 to 110 °C at 10 °C/min. Temperature calibration and calibration coefficient *E* for the DSC cell were determined using weighed samples of indium over the scanning range 25–200 °C. The reference pan contained an identical ovalbumin solution that was previously heat-denatured in the same temperature range as suggested by de Wit (1981). Rescanning the cooled ovalbumin showed no denaturation peak, suggesting irreversible denaturation (Patel et al., 1990). The cell was flushed with nitrogen at 50 mL min^{-1} to maintain an inert environment for all experiments. Onset denaturation temperature (T_o) was the temperature at which a change in slope of the curve first occurred, and T_d was defined as the endothermic peak temperature determined by Du Pont 9900 General V 2.2A software program.

Dynamic Rheological Testing. Storage moduli (G') of ovalbumin solutions (80 mg/mL) at pH 3.0, 7.0, and 9.0 in 25 mm KH_2PO_4 were monitored during isothermal heating for 15 min using a Rheometrics Fluids spectrometer (Model 8400, Piscataway, NJ), equipped with a 1–100 g-cm torque transducer and a silicon oil circulation system controlled by a Nelsprit temperature programmer (Model MTP-6, Newington, NH). One and a half milliliters of ovalbumin solution was placed between the cone and plate geometry (radius 12.5 mm, 0.02° cone angle and 50-μm gap) and equilibrated for 5 min before heating. The G' was recorded continuously at a fixed frequency of 1 rad/s and strain of 1.0%. Limits of constant viscoelasticity were determined by conducting frequency (0.1–100 rad/s) and strain sweeps (0.1–100%) in preliminary experiments.

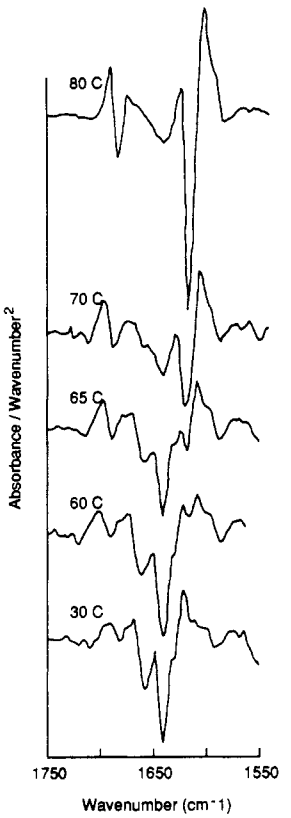

Figure 1. Second-derivative infrared spectra from 1550 to 1750 cm^{-1} of 1.75% *S*-ovalbumin in 25 mM KD_2PO_4, pD 3.0, heated for 15 min at the indicated temperatures.

Statistical Analyses. Basic statistics and two-way analysis of variance (replication × treatment) on a completely randomized design was determined using MSTAT software (version C, Michigan State University, East Lansing, MI). Tukey's honestly significant difference test ($P < 0.05$) was used to calculate significant differences between means. Differential scanning calorimetry and rheological experiments were performed in duplicate.

RESULTS AND DISCUSSION

Secondary Structure of Native *S*-Ovalbumin. Five amide I component bands were observed for *S*-ovalbumin at pD 3.0 (Figure 1) with peaks at 1638.2 (3_{10}-helix or β-sheet), 1656.3 (α-helix), 1682.5 (turn) and two shoulders, 1625 (β-sheet), and 1669.6 cm^{-1} (turn). There is strong evidence from other protein studies suggesting the band at 1639 cm^{-1} represents 3_{10}-helix (Acharya et al., 1989; Byler and Purcell, 1989; Prestrelski et al., 1991). However, in other proteins the band at 1639 cm^{-1} has been assigned to β-sheet (Dong et al., 1990). Some 3_{10}-helices were reported in the secondary structure of ovalbumin determined by X-ray crystallography (Stein et al., 1991); however, no 3_{10}-helix was identified by Raman spectroscopy (Painter and Koenig, 1976; Kint and Tominatsu, 1979).

The second-derivative FTIR spectrum of native *S*-ovalbumin at pD 7.0 exhibited four amide I peaks and a shoulder (Figure 2). The spectrum was similar to that of *S*-ovalbumin at pD 3.0. A distinguishable peak rather than a shoulder was observed at 1627.5 cm^{-1} (β-sheet). Peak intensity at 1638 cm^{-1} (3_{10}-helix or β-sheet) at pD 7.0 was larger than at pD 3.0. However, ovalbumin at pD 3.0 exhibited greater peak intensities at 1656 (α-helix) and 1681 cm^{-1} (turn) than at pD 7.0. These results are in contrast to those of Koseki et al. (1988), who reported similar CD curves for ovalbumin at pH 2.2 and 7.0.

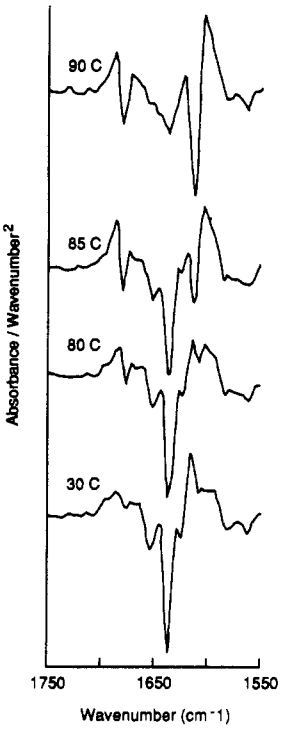

Figure 2. Second-derivative infrared spectra from 1550 to 1750 cm^{-1} of 1.75% S-ovalbumin in 25 mM KD_2PO_4, pD 7.0, heated for 15 min at the indicated temperatures.

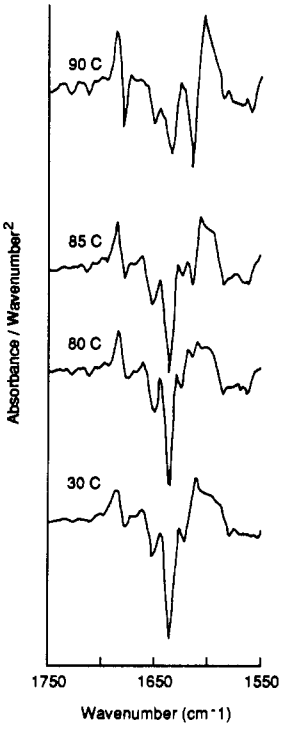

Figure 3. Second-derivative infrared spectra from 1550 to 1750 cm^{-1} of 1.75% S-ovalbumin in 25 mM KD_2PO_4, pD 9.0, heated for 15 min at the indicated temperatures.

The second-derivative FTIR spectrum of native S-ovalbumin at pD 9.0 exhibited four amide I peaks and a shoulder similar to the spectrum at pD 7.0 (Figure 3). The band representing 3_{10}-helix was larger at pD 9.0 than at pD 3.0, and the peak intensity representing α-helix was smaller at pD 9.0 than at pD 3.0.

Effect of Heating on Secondary Structure. Increasing the temperature shifted band frequencies of ovalbumin by 2–3 cm^{-1}. Peaks at 1637.2 (3_{10}-helix or β-sheet) and 1654.8 cm^{-1} (α-helix) decreased in intensity when S-ovalbumin, pD 3.0, was heated to 65 °C and above. No β-sheet structure was observed at 1625 cm^{-1}. The 1658.2-cm^{-1} (α-helix) peak decreased to a shoulder at 70 °C, indicating uncoiling of helical structure. The S-ovalbumin spectrum at 80 °C, pD 3.0, was similar to the 70 °C spectrum except the band at 1641.7 cm^{-1} (3_{10}-helix) was broader and the band at 1657.8 cm^{-1} (α-helix or β-sheet) was not observed. Because of the broadness of the peak at 1641.7 cm^{-1}, smaller peaks representing disordered structure (1645 cm^{-1}) and α-helix (1657.8 cm^{-1}) may have been masked.

S-Ovalbumin, pD 3.0, heated to 60 °C had increased band intensities at 1614 and 1682.5 cm^{-1} compared to ovalbumin at 30 °C. The peaks increased in intensity as ovalbumin heating temperature increased from 60 to 80 °C. In native proteins, the band at 1684 cm^{-1} has been identified as a type III turn. Type III turns have been identified in the crystal structure of ovalbumin (Stein et al., 1991); however, it is not clear why type III turns should increase on heating. Byler and Purcell (1989) observed the appearance of sharp peaks at 1614 and 1684 cm^{-1} when β-lactoglobulin and bovine serum albumin were heated to 80 and 75 °C, respectively. Clark et al. (1981) observed similar bands using infrared and Raman spectroscopy when several globular proteins were heated to form gels. Painter and Koenig (1976) reported the formation of intermolecular β-sheets upon thermal denaturation of ovalbumin when measured by Raman spectroscopy. The researchers (Byler and Purcell, 1989; Clark et al., 1981) attributed the bands to formation of hydrogen bonds caused by cross-linking β-strands during heat-induced gelation. Mine et al. (1990) and Kato and Takagi (1988) reported an increase in β-sheet structure as observed by CD when egg white protein or ovalbumin solutions were heated.

S-Ovalbumin, pD 7.0, heated to 80 °C and above (Figure 2) exhibited an increase in band intensity at 1613.4 and 1681.6 cm^{-1}, suggesting hydrogen bonding of β-sheet. Decreases in band intensity at 1638.7 (3_{10}-helix or β-sheet), 1655.8 (α-helix), and 1627.1 cm^{-1} (β-sheet) of ovalbumin heated to 80 and 85 °C were observed when compared to S-ovalbumin at 30 °C. A new band at 1670.9 cm^{-1} was observed which was identified as a turn (Byler and Susi, 1988).

S-Ovalbumin, pD 7.0, heated to 90 °C showed an increased band intensity at 1614.9 and 1682.5 cm^{-1}, and the band at 1627.1 cm^{-1} (β-sheet) was not observed. A decrease in band intensity at 1639.7 (3_{10}-helix or β-sheet) and 1657.7 cm^{-1} (α-helix) was observed compared to the 85 °C treatment.

S-Ovalbumin, pD 9.0, heated to 80 °C exhibited a new band at 1614.4 cm^{-1}, associated with hydrogen bonding (Byler and Purcell, 1989), which increased in intensity as heating temperature was increased. When S-ovalbumin was heated to 80 or 85 °C, a slight decrease was observed in the band at 1653.8 cm^{-1} (α-helix) and no changes in band intensities were exhibited at 1627.1 (β-sheet), 1636.8 (3_{10}-helix or β-sheet), 1670.9 (turn), and 1680.6 cm^{-1} (type III turn) when compared to the protein at 30 °C. The band intensity at 1683.5 cm^{-1} increased at 90 °C, suggesting the formation of hydrogen-bonded β-sheets. Band intensities at 1636.3 (3_{10}-helix or β-sheets) and 1653.8 cm^{-1} (α-helix) decreased, and those at 1624.6 (β-sheet) and 1670.9 cm^{-1} (turn) were not present when S-ovalbumin was heated to 90 °C.

Substitution of deuterium for hydrogen will affect the strength of hydrogen bonds and might alter protein

Table I. Influence of pH on Onset Temperature (T_o) and Thermal Denaturation Temperature (T_d) of S-Ovalbumin Solutions (80 mg/mL) As Determined by Differential Scanning Calorimetry

pH	T_o,[a] °C	T_d,[a] °C
3.0	68.0[a]	80.5[a]
7.0	83.6[b]	90.6[b]
9.0	82.4[b]	90.6[b]

[a] Means within columns followed by the same letter are not different ($P > 0.05$).

Table II. Storage Moduli of S-Ovalbumin Solutions (80 mg/mL) Heated Isothermally at Indicated Temperature for 15 min

	pH[a]		
temp, °C	3.0	7.0	9.0
65	16[a]		
70	35[b]		
80	239[c]	38[a]	26[a]
85		106[b]	199[b]
90		285[c]	382[c]

[a] Means within columns followed by the same letter are not different ($P > 0.05$).

stability (Calvin et al., 1959). Even with this potential limitation, some correlations were observed between heat-induced changes in secondary structure of ovalbumin by FTIR in D_2O and denaturation temperatures and storage moduli in H_2O. The T_o values of S-ovalbumin as determined by DSC at pH 3.0, 7.0, and 9.0 were 68.0, 83.6, and 82.4 °C, respectively (Table I). Few changes in S-ovalbumin secondary structure as measured by FTIR were observed below these temperatures. Decreases in the quantity of β-sheet, α-helix, and 3_{10}-helix in the secondary structure of S-ovalbumin were observed when the protein was heated to temperatures between the T_o and T_d at each pH.

Temperatures at which bands at 1614 and 1684 cm^{-1} increased in amplitude corresponded to increases in gel strength of S-ovalbumin as measured by storage modulus (Table II), suggesting the importance of hydrogen bonds to gel formation. At pH 3.0, the intensity of bands representing hydrogen bonding of β-sheet and storage moduli increased when S-ovalbumin heating temperature was increased from 70 to 80 °C. Similar changes in secondary structure and storage moduli of S-ovalbumin gels occurred at pH 7.0 and 9.0; however, these changes occurred at higher temperatures.

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