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Do Sulfhydryl Groups Affect Aggregation and Gelation Properties of Ovalbumin?

KERENSA BROERSEN,*,†,‡ ANNEMARIE M. M. VAN TEEFFELEN,‡ ANNEKE VRIES,‡ ALPHONS G. J. VORAGEN,‡ ROB J. HAMER,†,‡ AND HARMEN H. J. DE JONGH†,§

Wageningen Centre for Food Sciences, Diedenweg 20, P.O. Box 557, 6700 AN Wageningen, Laboratory for Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6700 EV Wageningen, and TNO Quality of Life, Utrechtseweg 48, 3704 HE Zeist, The Netherlands

The aim of this work is to evaluate the impact of sulfhydryl groups on ovalbumin aggregation and gelation. Ovalbumin was chemically modified to add sulfhydryl groups in various degrees. The rate of aggregation was not affected by the introduction of sulfhydryl groups, and disulfide bond formation was preceded by physical interactions. Hence, disulfide interactions may not be the driving force for the aggregation of ovalbumin. Investigation of the aggregates and gels by electron microscopy and rheology suggested that a critical number of sulfhydryl groups can be introduced beyond which the microstructure of the aggregates transforms from fibrillar into amorphous. Rheological studies further suggested that covalent networks, once formed, do not have the possibility to rearrange, reducing the possibility to attain a stronger network. These results show that, even though aggregation of ovalbumin may be primarily driven by physical interactions, formed disulfide bonds are important to determine the resulting aggregate morphology and rheological properties.

KEYWORDS: Ovalbumin; aggregation; gel properties; sulfhydryl groups; disulfide bonds; large and small deformation rheology; cryo-TEM

INTRODUCTION

Heat treatment has considerable implications for the behavior of proteins in food systems (*1*). One example of the consequences of heat processing is protein aggregation, which is a major determinant for the structural properties of food products. Egg white proteins are used frequently in the food industry for this purpose (*2*). Ovalbumin is the most abundant protein of egg white and therefore contributes to the functional properties of egg white protein preparations (*2*). Ovalbumin has one disulfide bond and four sulfhydryl groups buried in the interior of the protein (*3*).

A first step in the aggregation process is the (partial) unfolding of protein molecules upon heating. The conformational changes that are associated with unfolding result, among other changes, in solvent exposure of hydrophobic residues and sulfhydryl groups. Subsequent aggregation can involve noncovalent or intermolecular disulfide (covalent) interactions (*4*). At high protein concentrations, extensive aggregation can finally lead to the formation of a gel network. Gel networks are typically formed when the protein concentration exceeds a critical gelation concentration and the protein aggregates form a three-dimensional network (*5*). Aggregate and gel network formation are thus the result of a combination of hydrophobic and electrostatic (noncovalent) interactions, and in some cases, disulfide bonds (covalent interactions) can be present (*1*, *2*, *6*). All of these types of interactions can contribute to the rheological properties of the gel networks (*1*, *2*, *6*). In the past, kinetic models have been proposed to explain the formation of disulfide-linked aggregates for some proteins through a disulfide−sulfhydryl exchange reaction (*7*). This model suggests that the sulfhydryl group acts as an oxidizing agent to the intramolecular disulfide bond leading to the reduction of the disulfide bond. This suggestion is based on the observation that, upon disulfide linked aggregation, the total number of sulfhydryl groups is not affected. The resulting liberated sulfhydryl group may then undergo disulfide bridging with a sulfhydryl group of a proximate unfolded polypeptide chain. This reaction then proceeds further in analogy with a radical polymerization reaction (*8*). Clear evidence on the relative contribution of this mechanism to the aggregation and gelation processes has, however, not been provided up to now as other, noncovalent forces have been identified that potentially support the development of gel stiffness (*9*); clearly, the complex process of gel formation thus employs multiple types of interactions.

In the past, various approaches have been employed to evaluate the importance of sulfhydryl groups in the aggregation process including the reduction or chemical blockage of sulfhydryl groups (*10*−*13*) as well as activation of sulfhydryl

* To whom correspondence should be addressed. Present address: MRC Laboratory of Molecular Biology, Neurobiology Division, Hills Road, Cambridge CB2 2QH, England. Tel: +44(0)1223 402316. Fax: +44(0)-1223 402310. E-mail: broersen@mrc-lmb.cam.ac.uk.
† Wageningen Centre for Food Sciences.
‡ Wageningen University and Research Centre.
§ TNO Quality of Life.

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groups by unfolding of the protein (*14*−*16*). However, as protein unfolding simultaneously leads to exposure of hydrophobic regions, using (partially) unfolded molecules may not provide direct information on the effect of sulfhydryl groups. Also, the presence of chemicals such as DTT and NEM is known to affect the aggregation properties other than only by means of interruption of disulfide bond formation (*17*). Our approach involving the covalent attachment of acetylthiogroups that can be converted into reactive sulfhydryls on command through the cleavage of the acetyl group to the surface of folded protein molecules enables us to investigate the relative importance of sulfhydryl groups in the formation of aggregates and gel networks while maintaining the protein tertiary structure and other protein characteristics that potentially affect the aggregation process. This allows a direct comparison of the effect of the introduced sulfhydryl groups as opposed to the combined effect of covalent and noncovalent interactions. As such, the approach of chemical introduction of additional sulfhydryl groups is used in this work to investigate the role of these sulfhydryl groups in the process of aggregation formation, aggregation kinetics, and gel formation.

MATERIALS AND METHODS

Protein Purification and Modification. *Isolation and Purification.* Ovalbumin was purified from fresh hen eggs using a procedure based on previously described purification protocols (*18*). The protein solutions were dialyzed, freeze-dried, and stored at −20 °C until use. The purity was over 98% as determined using densitometric analysis of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) traces.

Modification. Acetylthio groups were introduced on the surface of the protein by the reaction between primary amino groups of lysine and S-acetylmercaptosuccinic anhydride (S-AMSA, Sigma) according to a previously described procedure (*19*). To vary the degree of modification, S-AMSA was added to the protein in ratios varying from 0.1:1 to 1.2:1 (S-AMSA: lysines). The acetylthiolation was performed at 25 °C and pH 8.0 for 24 h and a protein concentration of 25 mg/mL. After the incubation, the solutions were dialyzed extensively against demineralized water at 4 °C and subsequently freeze-dried and stored at −20 °C until use.

Fractionation. To obtain series of more defined ovalbumin variants, ion exchange chromatography (Biopilot, Pharmacia, MN) was used with a Source Q 900 mL column. A 200 mL amount of a 5 mg/mL protein solution in 10 mM sodium phosphate buffer was applied to the column at a flow rate of 15 mL/min and eluted using a gradient from 0.08 M NaCl, pH 6.0, to 1.00 M NaCl, pH 6.0.

Deblocking. The fractions obtained from ion exchange chromatography were divided into two batches. The acetyl group was cleaved off from the modified proteins in one of the batches, called SH ovalbumin, by the addition of 1.0 M hydroxylamine hydrochloride (Sigma) at pH 7.0 and 25 °C. The mixture was incubated for 1 h and then dialyzed extensively against demineralized water at 4 °C. The untreated batch contained proteins with a blocked sulfhydryl group, called SX ovalbumin, and was left untreated and contained an acetylthio group.

Protein Characterization. *Chromogenic OPA Assay.* The degree of modification of the primary amino groups was determined indirectly by a chromogenic assay based on the specific reaction between *ortho*-pthaldialdehyde (OPA, Sigma) and free primary amino groups in proteins (*20*). All measurements were performed in duplicate and corrected for a protein-free sample. The protein concentration was determined by the adsorption at 280 nm using an extinction coefficient of 0.712 mL mg^{-1} cm^{-1}.

Determination of Free Sulfhydryl Groups. Ellman's reagent was used to determine the number of free thiol groups. The reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB (Sigma), reacted with free thiol groups on the protein (*21*). Cysteine (L-cysteine HCl monohydrate, Sigma) was used as a calibration standard. The DTNB, 4.8 mg, was dissolved in 3.5 mL of Tris-HCl buffer (pH 8.0 and 2% SDS). Fifty microliters of DTNB solution was added to 250 μL of 5 mg/mL protein solution. The solution was mixed and incubated for 20 min at 25 °C. Then, the absorbance was measured at 412 nm. The calibration curve, obtained by using various dilutions of a 0.50 mM cysteine solution, provided an extinction coefficient of sulfhydryl groups of 13425 M^{-1} cm^{-1}. Previously, it was verified using the sulfhydryl-disulfide exchange index (SEI index) (*22*) that the sulfhydryl groups introduced were chemically reactive (*23*).

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra of 0.1 mg/mL protein solutions in 10 mM phosphate buffer, pH 7.0, were recorded at 25 °C in the spectral range from 190 to 260 nm with a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) using a quartz cuvette with an optical path of 0.1 cm. The spectral resolution was 0.5 nm, the scan speed was 100 nm/min, and the response time was 0.125 s with a bandwidth of 1 nm. Sixteen scans were accumulated and averaged. The spectra were corrected for the corresponding protein-free sample. Near-UV CD spectra were obtained of 1.0 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) at 25 °C in the spectral range of 250−350 nm in quartz cuvettes with an optical path of 1 cm. A spectral resolution of 0.5 nm was used, and a scan speed of 100 nm/min, a response time of 0.125 s, and a bandwidth of 1 nm were used. Sixteen scans were accumulated and averaged, and the spectra were corrected for a protein-free sample.

Fluorescence Spectroscopy. Fluorescence spectroscopy was used to obtain information on the impact of the modification procedure on the tertiary structure of ovalbumin. Emission spectra of 5 μg/mL protein in a 10 mM phosphate buffer (pH 7.0) were analyzed using a Perkin-Elmer Luminescence Spectrometer (LS50B, United States) at 20 °C. Quartz cuvettes with an optical path of 1 cm were used. The excitation and emission slit widths were 5 nm, and a scan speed of 120 nm/min was used. Spectra were obtained from 300 to 400 nm at excitation wavelengths of 295 and 274 nm. All spectra were performed in duplicate and corrected for the corresponding protein-free sample.

Isoelectric Focusing (IEF). The apparent isoelectric points of the ovalbumin variants were determined using the Phast System (Pharmacia, United States). Four microliters of 1.0 mg/mL protein solutions was applied to IEF gels with a pH gradient ranging from 2.5 to 6.5 (Pharmacia) and from 3 to 10 (Pharmacia). The gels were fixed with 20% trichloric acid, stained using Coomassie Brilliant blue (R-250, Merck), and destained in 30% methanol/10% acetic acid.

Differential Scanning Calorimetry (DSC). Amounts of 4.0 mg/mL protein solutions in 10 mM phosphate buffer, pH 7.0, were sealed in the cell of a VP-DSC MicroCalorimeter (MicroCal Inc., Northampton, United States). A 10 mM phosphate buffer was used as a reference sample. The heat flow was recorded in separate duplicates from 25 to 110 °C at a heating rate of 1 °C/min. The data were analyzed using the MicroCal Origin software.

Aggregation Kinetics. The aggregation kinetics of the ovalbumin variants were tested by incubation at 2 °C below the gel temperature. The gel temperature was defined as the temperature at which G' significantly deviated from G'' in rheology measurements. Unmodified, SX8 (ovalbumin with eight acetylthiogroups) and SH8 (ovalbumin with eight additional sulfhydryl groups) ovalbumin were incubated at their individual thermal transition temperatures determined by DSC, 72, 76, and 64 °C, respectively, at a concentration of 3 mg/mL in 50 mM phosphate buffer at pH 7.0. Fifty microliter aliquots were withdrawn at various time intervals from 0 to 6 h. Alternatively, all ovalbumin variants were incubated at 70 °C at a concentration of 5 mg/mL in 50 mM phosphate buffer, pH 7.0, and 0.15 M NaCl and aliquots were withdrawn at a series of time intervals up to 380 min. After sampling, the solutions were diluted directly into equal volumes of SDS−PAGE sample buffer containing 2% SDS and 30 mM NEM and cooled on ice. After cooling, the samples were applied to an SDS−PAGE gel as described below.

SDS−PAGE. SDS−PAGE was performed according to Laemmli (*24*). A 15% (w/v) acrylamide separating gel and a 4% (w/v) acrylamide stacking gel containing 0.1% SDS (w/v) were run using a Mini-PROTEAN II Electrophoresis Cell (Biorad, United States). Samples of 0.1 mM protein were prepared in sample buffer containing 10% SDS (w/v) and 1.25% (v/v/) β-mercaptoethanol. Gels were stained with

Coomassie Brilliant Blue R-250 and destained with methanol:acetic acid:water (30:10:60) (v/v/v) acetic acid in water. The fractions of monomeric and polymeric material were estimated using densitometric analysis. The accuracy of this analysis was estimated as 5% derived from triplicate measurements and subsequent densitometric analysis.

Aggregate Morphology. *Transmission Electron Microscopy (TEM).* Amounts of 20 mg/mL protein solutions in 10 mM phosphate buffer, pH 7.0, in the absence and presence of 0.15 M NaCl were prepared, to correct for the introduced net charge by the additional carboxyl groups, heated for 1 h at 90 °C, and subsequently cooled to 20 °C. The solutions were diluted to 5 mg/mL and placed on a perforated carbon film, supported by a 200 mesh copper grid, and subsequently blotted. The liquid film was vitrified by rapidly plunging the grid into liquid propane at a temperature of −170 °C. The specimens were stored in liquid nitrogen until transfer into the cryoholder. The images were recorded digitally using a Philips CM12 transmission electron microscope (The Netherlands) operating at 80 kV with a Gatan 791 CCD camera using Digital Micrograph software.

Gel Properties. *Large Deformation Rheology.* A 250 μL amount of a 50 mg/mL protein solution in a 50 mM phosphate buffer at pH 7.0 was transferred to a microtiter plate, sealed, and incubated for 2 h in a water bath at 90 °C. Next, the titer plate was cooled to 20 °C and the gels were tested for fracture using a TA-XT2i Texture Analyzer (Stable Micro Systems, United Kingdom) and a measuring probe with a diameter of 6 mm. The downward speed was 2.0 mm/s until the threshold force exceeded 0.010 N. Then, the probe speed was adjusted to 0.2 mm/s from which point it penetrated the gel to a distance of 5.0 mm. The measurements were performed in triplicate. The Young's modulus E (Pa), or relative change in stress as a function of change in strain, was calculated from the resulting stress−strain curves by the definition $E = [d\sigma/d\epsilon]_\epsilon \rightarrow 0$, where $d\sigma$ is the change in the tensile stress and $d\epsilon$ is the change in the tensile strain (*25*).

Small Deformation Rheology. The viscoelastic properties of solutions with a range of protein concentrations from 30 to 80 mg/mL at pH 7.0 in the absence and presence of 0.15 M NaCl were determined using a Bohlin CVO controlled stress rheometer (Malvern, Worcestershire, United Kingdom) with a concentric cylinder geometry (C14). The samples were covered with paraffin oil to prevent solvent evaporation. The storage modulus (G') and loss modulus (G'') were determined with a target strain of 0.001 at an angular frequency of 1 Hz. The initial stress was aimed at 0.0706 Pa. The samples were heated from 20 to 90 °C at a heating rate of 1 °C/min. Then, the solutions were maintained at 90 °C for 120 min and subsequently cooled to 20 °C at a rate of 2 °C/min. At 20 °C, an oscillation sweep was performed during which the shear stress was increased from 0.0706 Pa to a maximum of 7000 Pa.

RESULTS

Preparation and Fractionation of Modified Ovalbumin Fractions. To modify ovalbumin, various ratios of S-AMSA:lysine were used to produce a series of ovalbumins with different numbers of additional sulfhydryl groups (**Table 1**). Using the OPA assay (sensitive to free amino groups) and the Ellman assay (sensitive to free thiol groups), it was found that the average number of additional sulfhydryl groups per ovalbumin molecule ranged from zero to 10 (**Table 1**). The results obtained from Ellman's assay are in agreement with the results from the OPA assay (**Table 1**). The average degree of modification increased with increasing the S-AMSA:lysine ratio as has been found before (*19*, *26*, *27*). It has previously been verified that chemically introduced sulfhydryl groups are active using the SEI (*23*). As a consequence of the reaction of lysine with S-AMSA, acetylthio groups are formed (*19*). The sulfur group is blocked by an acetyl group. Reaction of S-AMSA with the lysines also results in the introduction of an additional carboxyl group. This additional carboxyl group can be used to estimate the degree of heterogeneity of the modification as well as to limit the heterogeneity of the formed proteins by fractionation using ion exchange chromatography. The batches produced with different S-AMSA:lysine ratios showed heterogeneity as was established by a significant broadening of the elution profiles obtained from ion exchange chromatography, as compared to unmodified ovalbumin (**Figure 1**). By fractionation using ion exchange chromatography, we were able to isolate more homogeneous fractions (±1 acetylthiogroup). The protecting acetyl groups were cleaved off by hydroxylamine hydrochloride to obtain reactive sulfhydryl groups just prior to usage in order to limit the extent of autoxidation of unprotected sulfhydryl groups as has before been reported (*28*). In doing so, we obtained ovalbumins within each degree of modification, which contained either acetylthio groups (SX) or (SH) sulfhydryl groups (**Table 1**). The fractions were annotated numbers that reflect the number of introduced sulfhydryl groups per protein molecule (e.g., SX8 or SH8).

The isoelectric point decreases with increasing degree of modification from pI 4.7 for unmodified ovalbumin down to pI 3.9 for SX10/SH10 (**Table 1**). The change in pI results from the parallel introduction of one carboxyl group with each

Table 1. Physicochemical Characteristics of Modified Ovalbumin Fractions[a]

	no. of sulfhydryl groups (Ellman assay)	no. of modified groups (OPA assay)	average degree of modification (%)	isoelectric point (IEF)	transition temp (°C, DSC) SX/SH
unmodified	0.9 ± 0.1	0	0	4.7	78.6
SX/SH1	1.8 ± 0.3	1.41 ± 0.02	7	4.4	ND/ND
SX/SH4	3.7 ± 0.4	3.9 ± 0.4	18	4.3	ND/ND
SX/SH6	5.4 ± 0.5	5.5 ± 0.1	26	4.2	73.9/72.7
SX/SH8	9.2 ± 0.9	8.1 ± 0.8	38	4.0	73.2/66.5
SX/SH10	10.1 ± 0.6	10.0 ± 0.3	47	3.9	ND/ND

[a] ND, not determined. Average degree of modification (% modified lysines of the total number of lysines available per ovalbumin molecule), isoelectric point, and thermal transition temperature.

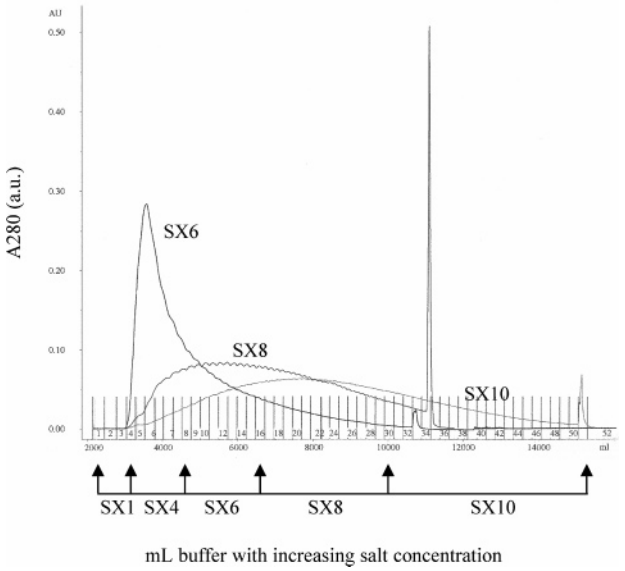

Figure 1. Fractionation of modified ovalbumin by ion exchange chromatography. The chromatograms shown represent the nonfractionated ovalbumin fractions incubated with various S-AMSA concentrations. The arrows at the bottom show which fractions were subsequently pooled to obtain more homogeneous fractions. Fractions 4–8, SX4; fractions 9–16, SX6; fractions 17–30, SX8; and fractions 31–50, SX10.

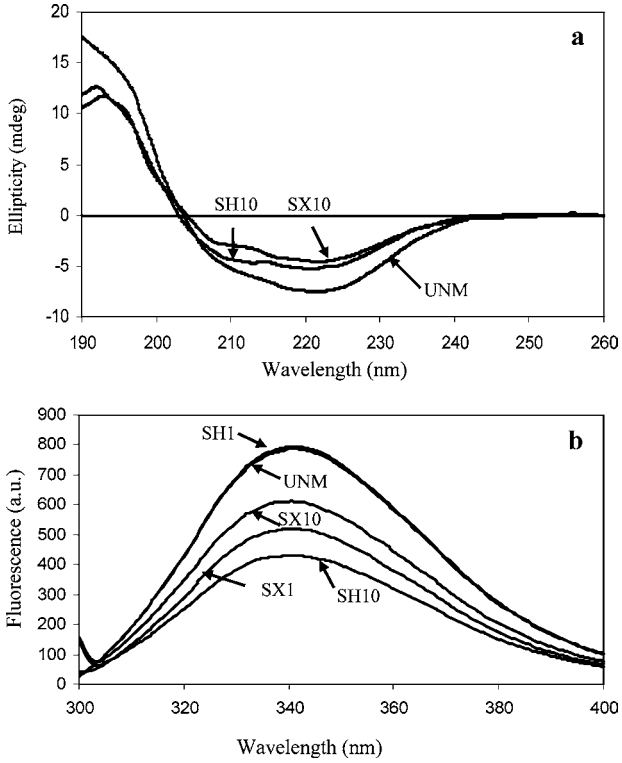

Figure 2. Structural characterization of ovalbumin fractions. (**a**) Far-UV CD spectra of unmodified, SX10, and SH10 ovalbumin. (**b**) Intrinsic tryptophan fluorescence spectra of unmodified, SH1, SX1, SH10, and SX10 ovalbumin.

additional sulfhydryl group and the concomitant loss of a positive charge of the reacted lysine.

Structural Characterization of Modified Ovalbumin Fractions. Fluorescence and far-UV CD spectra were recorded for all SX and SH fractions produced to determine the impact of the modification on the secondary and tertiary structure of ovalbumin. **Figure 2a** shows the far-UV CD spectra of unmodified, SX10, and SH10 ovalbumin, the latter two being the highest degrees of modification. Particularly in the intensity of the spectra, some changes can be observed. It was deduced from these spectra (**Figure 2a**) that the secondary structure was affected by the modification procedure. The far-UV CD spectra of the other ovalbumins did not provide indications for structural changes as compared to unmodified ovalbumin (results not shown). Analysis of the spectra using a previously described method (*29*) provided estimates of the relative contribution of random coil elements, which were, however, found not to differ significantly from the unmodified ovalbumin for the modified fractions. For example, the percentage of random coil calculated from the spectra varied between 24 and 27% random coil for the unmodified and modified ovalbumin variants.

Typical fluorescence spectra of unmodified ovalbumin, SH1, SX1, SH10, and SX10 are shown in **Figure 2b**. The intensity of the fluorescence shows a significant difference upon modification, which appeared not to be related to the degree of modification. It was also found that the wavelength with the maximum fluorescence intensity (340 nm) did not shift upon modification. This suggests that the tertiary structure of the ovalbumin fractions was retained. As a variation in fluorescence intensity may be the result of a different energy transfer efficiency between tryptophan and tyrosine, fluorescence data obtained at an excitation wavelength of 295 and 274 nm were compared. The results gave no indication that the globular structure of the ovalbumin fractions was affected by the

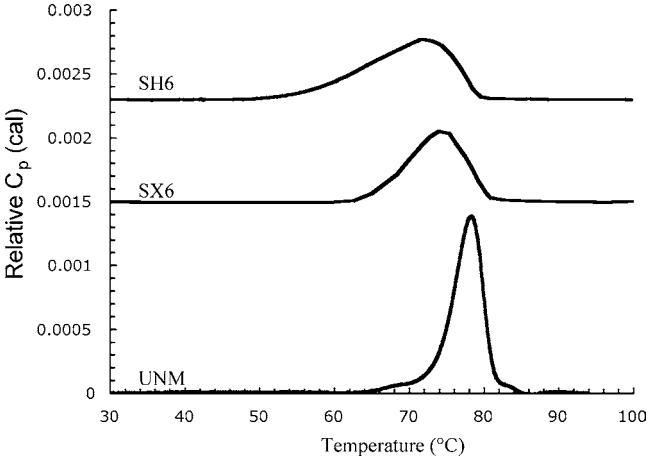

Figure 3. DSC of ovalbumin fractions. Heat capacity with increasing temperature of unmodified (UNM), SX6, and SH6 ovalbumin. The spectra have been horizontally displaced for clarity.

modification (results not shown). Near-UV CD spectra of the ovalbumin fractions also indicated that the tertiary structure was not affected upon modification (results not shown). From these results, it was concluded that introduction of 0−10 additional sulfhydryl groups did not result in a significant loss of structural properties of ovalbumin at a tertiary level.

The thermal transition temperatures of the SH/SX fractions were determined using DSC. The thermograms of unmodified, SX6, and SH6 ovalbumin are shown as examples in **Figure 3**. As illustrated in this figure, the variants are able to undergo an unfolding transition as can be deduced from the endothermic contribution at the thermal transition temperatures. This indicates that the modified ovalbumin variants contained significant structural integrity as was also confirmed by the CD and fluorescence data (**Figure 2**). The unmodified ovalbumin has a thermal transition temperature of 78.6 °C. The fraction with six acetylthiogroups has a thermal transition temperature of 73.9 °C while removal of the acetyl groups resulted in a lower thermal transition temperature of 72.7 °C. The introduction of eight groups resulted in a further reduction of the thermal transition temperature down to 66.5 °C (**Table 1**). It is possible that charge modification, such as the introduction of additional carboxyl groups, can significantly contribute to the observed thermostability effects.

Correction for Net Charge To Study Aggregation and Gelation. In the past, it has been verified that carboxyl groups can significantly affect the rate at which protein molecules aggregate (*30*, *31*). These two papers both show that calcium binding to carboxyl groups of the proteins investigated, α-synuclein and β-lactoglobulin, respectively, results in site-specific screening of surface charges, facilitating the aggregation process. It is thus likely that net charge can also affect the unfolding kinetics and aggregation and gelation properties of ovalbumin. The fractions with acetylthiogroups (SX) were therefore directly compared to the reactive fractions (SH) with the same degree of modification, thus correcting for the effect of net charge. Next to this, 0.15 M NaCl was added and found to be sufficient to counteract the side effect of the introduction of carboxyl groups in addition to sulfhydryl groups based on calculations using the equations developed by Wu et al. (*32*). For this reason, the aggregation and gelation experiments reported here have been carried out in the presence of these concentrations of NaCl.

Effect of Sulfhydryl Groups on the Microstructure of Aggregates. To study the effect of sulfhydryl groups on the morphology of the formed aggregates, TEM was used to obtain

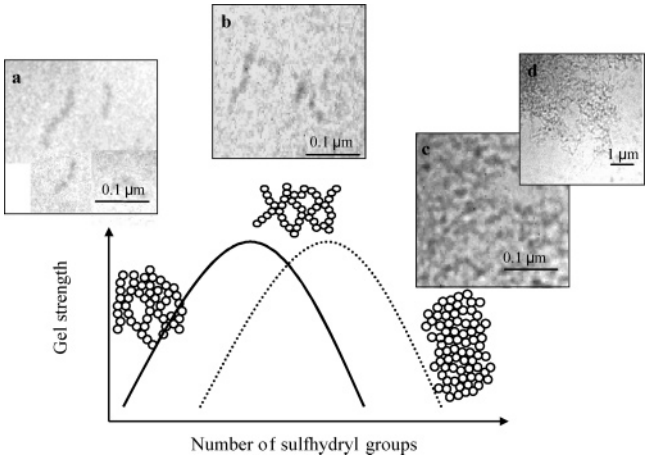

Figure 4. Model for aggregation and gelation of ovalbumin with additional sulfhydryl groups. (**a**) TEM micrograph of SX1 aggregates in the presence of 0.15 M NaCl, (**b**) TEM micrograph of SH4 aggregates in the presence of 0.15 M NaCl, (**c**) TEM micrograph of SH10 aggregates in the presence of 0.15 M NaCl, and (**d**) TEM micrograph of SH10 aggregates in the presence of 0.15 M NaCl. The lines represent the development of the gel strength of ovalbumin gels with increasing numbers of reactive sulfhydryl groups in the absence (continuous line) and presence of NaCl (interrupted line).

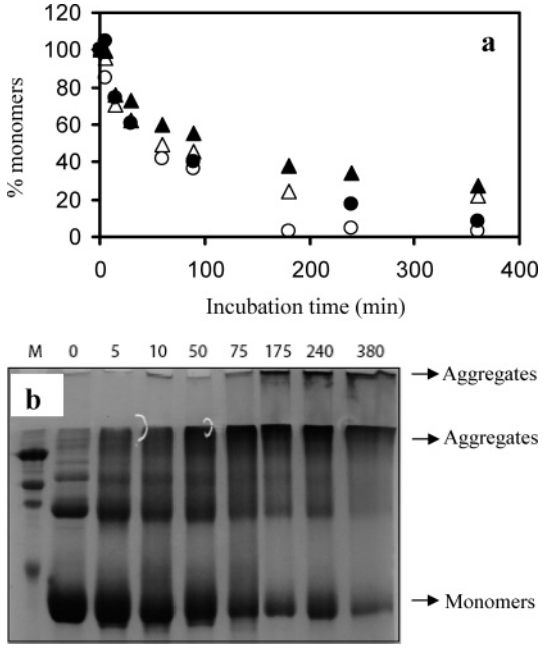

Figure 5. Aggregation kinetics of ovalbumin with different degrees of modification. (**a**) Disappearance of monomeric material as determined by SDS−PAGE with increasing incubation time in the presence of 0.15 M NaCl at elevated temperatures of SH ovalbumin (open symbols), SX ovalbumin (closed symbols), SX/SH10 (circles), and SX/SH8 (triangles). (**b**) SDS−PAGE of SH10 ovalbumin with increasing incubation time (mins) at elevated temperatures in the presence of 0.15 M NaCl.

information concerning the organization of aggregates (**Figure 4**). Aggregates of ovalbumin with the acetylthio groups are characterized by short and unbranched fibrillar aggregates with a homodisperse size distribution (**Figure 4a**). Aggregates produced under similar conditions but containing four reactive sulfhydryl groups are slightly longer, homogeneously distributed in size, and also unbranched (**Figure 4b**). Upon introducing 10 additional sulfhydryl groups, curved aggregates are formed with a high degree of branching (**Figure 4c**,**d**). Clearly, the degree of modification has an impact on the microstructure of the aggregates.

Effect of Sulfhydryl Groups on the Aggregation Kinetics. **Figure 5a** shows the aggregation kinetics for SX/SH8 and SX/SH10 ovalbumin in the presence of 0.15 M NaCl. Protein solutions were incubated at a concentration of 5 mg/mL at pH 7.0 and a temperature of 70 °C for various time intervals from 0 to 380 min. After cooling, the samples were applied to nonreducing SDS−PAGE to determine the content of disulfide bonds formed upon heating (example shown for SH10 in **Figure 5b**). It was found that the fraction of monomeric proteins declined with increasing incubation time due to aggregation (**Figure 5**). Comparing the rates of the loss of the monomeric protein fraction for SX8 and SH8 fractions showed only very slight differences in the rates of aggregation of sulfhydryl vs acetylthiogroups for both the SH/SX10 fractions as for the SH/SX8 fractions. For example, 50% of the SH8 molecules had aggregated after 60 min of incubation at elevated temperature, and after 100 min, only 50% of the SX8 molecules had aggregated.

The other fractions tested also showed similar results for the rate of aggregation when comparing acetylthio groups and sulfhydryl groups with the same degree of modification (results not shown).

To test if these results were affected by the difference in thermal transition temperatures, the aggregation kinetics of the SX8 and SH8 fractions were also compared at their individual thermal transition temperatures (at 66 °C for SH8 ovalbumin and at 73 °C for SX8 ovalbumin). It was found that under these conditions the aggregation rates found by electrophoresis were comparable for the SH8 and SX8 fraction (results not shown). The differences in thermal stability thus appear to explain the different aggregation rates rather than the different numbers of sulfhydryl groups available for the reaction.

Effect of Sulfhydryl Groups on Large Deformation Properties of Gels. Large deformation properties of gels from the various SX/SH fractions were determined by penetration analysis. From the force of the probe required to compress the gel, the Young's modulus was calculated, as an indication for gel stiffness (**Figure 6a**). The gel strength was defined as the threshold force at which the gel fractures (**Figure 6b**). As shown in **Figure 6**, the Young's modulus (**Figure 6a**) and the gel strength (**Figure 6b**) are both significantly affected by the presence of reactive sulfhydryl groups. The Young's modulus was significantly lower for gels with reactive sulfhydryl groups (approximately 200 Pa) as compared to gels with blocked sulfhydryl groups (ranging from 1300 to 1800 Pa) for all degrees of modification tested. At first sight, it was expected that addition of NaCl would screen the electrostatic repulsion introduced together with the addition of sulfhydryl groups, allowing stiffer gels to be formed. Surprisingly, the presence of 0.15 M NaCl did not result in an increased gel stiffness with reactive sulfhydryl groups (approximately 200 Pa) although the introduction of eight reactive sulfhydryl groups lead to an increase only at this specific degree of modification in the Young's modulus to 600 Pa. Even the introduction of 10 sulfhydryl groups did not result in a significantly higher Young's modulus. In contrast to this, for gels with blocked, nonreactive sulfhydryl groups in the presence of 0.15 M NaCl, the Young's modulus gradually increased from 800 to 1600 Pa with increasing degrees of modification for gels with blocked sulfhydryl groups.

The threshold force at which the gel fractures initially increases with increasing the number of reactive sulfhydryl

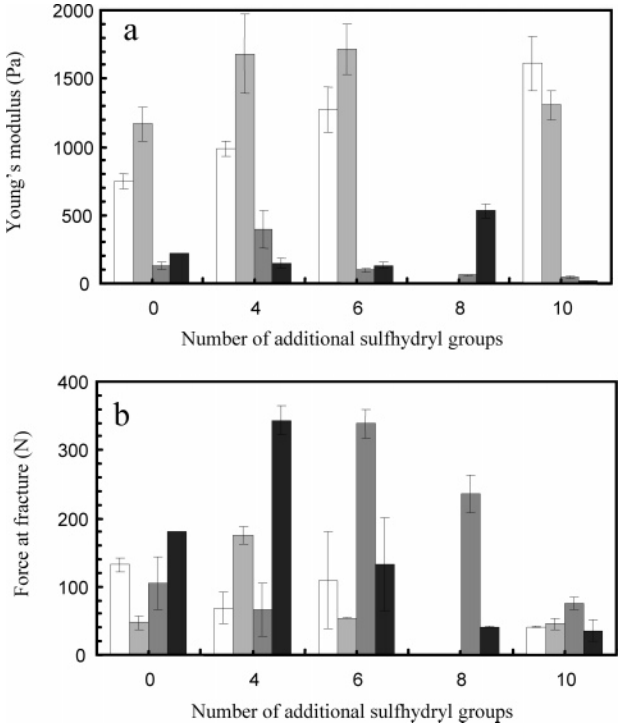

Figure 6. Large deformation rheology of ovalbumin gels. (**a**) Young's modulus. SH ovalbumin gels in the presence (black bars) and absence (dark gray bars) of 0.15 M NaCl and SX ovalbumin gels in the presence (white bars) and absence of 0.15 M NaCl (light gray bars). (**b**) Gel strength of ovalbumin gels as determined by applied force at which the gel network fractures. The legend is as for **Figure 6a**.

groups present on the ovalbumin molecule from 100 to 340 N (**Figure 6b**) upon introducing six reactive sulfhydryl groups in the absence of NaCl. In the presence of 0.15 M NaCl, an optimum of 350 N is reached upon introducing four reactive sulfhydryl groups and increasing the degree of modification further resulted in a decrease of the gel strength down to approximately 100 N. The gels with blocked sulfhydryl groups follow a similar trend as the gels with reactive sulfhydryl groups with a maximum gel strength upon introducing six sulfhydryl groups in the absence of NaCl and four sulfhydryl groups in the presence of NaCl, but the effect on gel strength appears smaller. The maximum threshold force at fracture is 170 N in the absence of NaCl and 120 N in the presence of NaCl.

Effect of Sulfhydryl Groups on the Small Deformation Properties of Gels. The development of the storage modulus (G') of ovalbumin solutions (in 50 mM phosphate buffer at pH 7.0) was monitored during a heating step (from 20 to 90 °C at 1 °C/min), a gelling step (constant temperature of 90 °C for 120 min), and a cooling step (from 90 to 20 °C at 1 °C/min). In **Figure 7a**, this is demonstrated for the SH4 and SX4 fractions. The gel point temperature can be determined by extrapolation of the rapid rise in G' upon increasing temperature (*33*). Upon heating the SH4 ovalbumin solution, G' increases from a temperature of 68 °C whereas G' of SX4 ovalbumin increases from a temperature of 75 °C. This finding is consistent with the observation that the thermal transition temperature differs between the SX and the SH fractions (example shown for SX6/SH6 in **Figure 3**). After this initial increase, G' continues increasing rapidly upon further heating and develops in a similar fashion for SX4 and SH4 ovalbumin gels (**Figure 7a**). During the gelling step, the ovalbumin gels slowly develop further as indicated by a further increase in G' at 90 °C. Upon cooling, G' increases rapidly to a G' of 444 ± 44 Pa for the

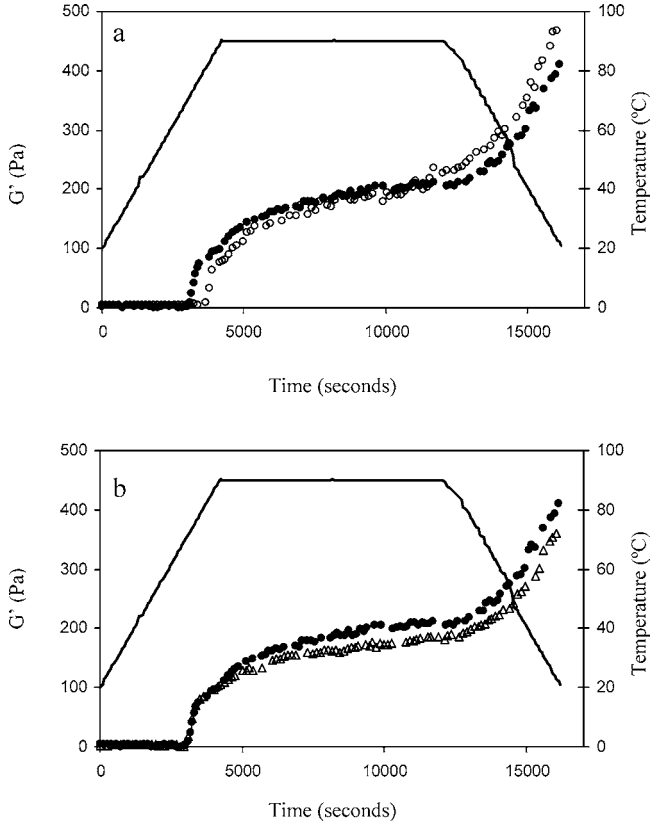

Figure 7. Small deformation rheology of ovalbumin gels. (**a**) Storage modulus G' as a function of time and temperature of SH4 (closed circles) and SX4 (open circles) gels in the absence of NaCl. (**b**) Storage modulus G' as a function of time and temperature of SH4 gels in the absence (closed circles) and in the presence of 0.15 M NaCl (open triangles). The continuous line represents the temperature at which the samples were incubated.

SH4 ovalbumin gel and 496 ± 2 Pa for the SX4 ovalbumin gel at 20 °C. Apparently, the unblocking of the sulfhydryl groups does not result in significantly stiffer gels as compared to the gels containing blocked sulfhydryl groups.

The effect of the introduction of reactive sulfhydryl groups was also investigated in the presence of 0.15 M NaCl (**Figure 7b**). It was found that the addition of NaCl does not result in an increased G' (376 ± 10 Pa) for gels with reactive sulfhydryl groups and rather resulted in a slightly decreased gel stiffness. This was the case for all of the gels tested within the entire range of modification degrees (results not shown).

To test whether the gels prepared from the ovalbumin variants were constructed of covalent or noncovalent networks, the gels prepared were dissolved in various solvents. It was found that gels consisting of acetylthio groups were well soluble in SDS only (in the absence of β-mercaptoethanol). This indicates that the aggregates were primarily constructed of noncovalent interactions. In contrast, aggregates containing reactive sulfhydryl groups were only partially dissolved in SDS. Complete dissolving required the presence of β-mercaptoethanol indicating a combined contribution of covalent and noncovalent interactions to the network (results not shown).

DISCUSSION

Approach. This work aims to investigate the contribution of disulfide bonds to the aggregation and gelation processes of ovalbumin. Aggregation is the result of attractive forces exceeding repulsive forces. Attraction is governed by a com-

bination of noncovalent and covalent (disulfide bonds) interactions, and the repulsive forces are often electrostatic or steric in nature. In the past, various reports have underlined the relevance of sulfhydryl groups and disulfide bonds to protein−protein interactions or protein−volatile flavor interactions (*2*, *34*). Heat treatment of ovalbumin results in the exposure of additional sulfhydryl groups, which subsequently can be available for aggregation (*35*). The introduction of additional sulfhydryl groups at the surface of ovalbumin was therefore hypothesized to enhance the opportunity for covalent interactions thus affecting aggregation kinetics and gelation properties. To this point, ovalbumin fractions were produced containing a range from zero to 10 additional sulfhydryl groups. The reactivity of the introduced sulfhydryl groups was tested (*23*), and even though the modification significantly affected the conformational properties of the proteins at a secondary structure level, their ability to unfold at elevated temperatures was retained suggesting that the proteins were still folded (**Table 1** and **Figures 2** and **3**). The results for ovalbumin with acetylthio groups or sulfhydryl groups were compared as this, next to the reduction of electrostatic repulsion by the addition of salt, allowed us to draw conclusions on the contribution of sulfhydryl groups to disulfide bonds in the aggregation and gelation process while eliminating other side effects related to the modification procedure.

Impact of Sulfhydryl Groups on Aggregation. The attachment of reactive sulfhydryl groups to the surface of ovalbumin molecules did not result in spontaneous formation of covalently linked aggregates at room temperature, even when increasing the concentration to 200 mg/mL (*23*) even though the sulfhydryl groups were already exposed and available for cross-linking. This implies that other attractive forces are required to enable sulfhydryl groups to remain in close proximity of each other for a certain time scale sufficient for disulfide interactions to occur and also that disulfide bond formation may not be a driving force for aggregation.

At elevated temperatures, the ovalbumin fractions with reactive sulfhydryl groups showed a comparable rate of aggregation as the ovalbumin fractions with nonreactive acetylthiogroups, suggesting that the rate of aggregation was not significantly affected by the presence of reactive sulfhydryl groups (**Figure 5**). Apparently, sulfhydryl groups do not affect the rate of aggregation for ovalbumin; therefore, it is suggested that disulfide linkage is not the driving force for aggregation. The question remains how generic this observation is for other proteins. It has been shown before, however, that noncovalent aggregation precedes the disulfide cross-link reaction for ovalbumin and whey protein isolate as well as for vicilin (*36*, *37*).

Impact of Sulfhydryl Groups on Gelation. In contrast to the negligible effect on the rate of aggregation, the gel network properties appeared to be significantly affected by the introduction of reactive sulfhydryl groups. Sun and Hayakawa (*2*) have shown before that ovalbumin gels become more rigid when the pH is increased to pH 7.0−8.5. Considering the increased deprotonation of sulfhydryl groups at this pH range, it can be expected that the increased rigidity of the formed gels observed by Sun and Hayakawa (*2*) are the likely effect of an improved reactivity of the sulfhydryl groups. These results suggest a significant contribution of sulfhydryl groups to the obtained gel rigidity. We showed that the Young's modulus was significantly higher for gels containing only blocked and thus no additional sulfhydryl groups as compared to gels containing reactive sulfhydryl groups (**Figure 6a**). As for the gel strength, which was defined as the threshold force upon which the network fractures, it was found that optimal degrees of modification exist for gels containing reactive sulfhydryl groups. This optimum was also affected by the presence of NaCl (**Figure 6b**). It is thus clear from the results presented in **Figure 6a**,**b** that the number of sulfhydryl groups available on the protein molecule does not readily predict the resulting gel stiffness or gel strength. These results can potentially be explained as follows. By dissolving the formed gel networks in a variety of reagents containing either SDS or a combination of SDS and β-mercaptoethanol, it was found that gels consisting of blocked sulfhydryl groups were well soluble in SDS only. This confirmed that the gel network was primarily constructed of noncovalent interactions. Gels containing reactive sulfhydryl groups were partially dissolved in SDS and dissolved totally only in the presence of β-mercaptoethanol indicating a combined contribution of covalent and noncovalent interactions to the network (results not shown). The permanent character of the disulfide bond, due to its high bond energy, does not provide rotational freedom in the gel network for hydrophobic interactions to rearrange themselves into a structure to optimize the gel strength. Also, as provided for in the equation below developed by van Vliet et al. in 1985 (*38*), a number of other parameters, apart from the number of potential disulfide bonds to be formed, describe the stiffness of a gel.

$$G = C\,N \cdot \mathrm{d}^2 F/\mathrm{d}x^2$$

where G is the modulus, C is a characteristic length determined by the geometry of the network, N is the number of strands per unit area bearing the stress, dF is the change in Gibbs free energy when the elements are moved apart over a distance dx. The geometry of the formed aggregates (C) affects the resulting modulus strongly and, as was shown by cryo-TEM of the gels, the curvature of the aggregates was significantly affected by the presence of sulfhydryl groups (**Figure 4**). More curvature generally leads to a lower gel stiffness as compared to more linear aggregates as is related in the model depicted in **Figure 4**. Also, the number of interactions importantly determines the resulting gel stiffness. Rearrangement of hydrophobic interactions upon heating the gel may provide additional means of bearing the force applied to the gel, a possibility that is disabled upon covalently and thus more or less permanently, linking gels by disulfide interactions. The final gel strength is affected by the number of possible interactions and may induce an actual higher shear stress for blocked vs unblocked variants by this mechanism. The Gibbs free energy content of individual hydrophobic interactions may be lower as compared to the strong nature of a covalent disulfide bond. However, apparently, the gel stiffness is affected significantly more by the large number of hydrophobic interactions as compared to the limited availability of sulfhydryl groups to undergo disulfide interactions in the modified ovalbumins. Similar indications have been reported previously, but these rearrangements did not affect the microstructure of the gel network (*37*, *39*). **Figure 4** shows schematically how the effect of sulfhydryl groups on gel network properties can be depicted. At a low degree of substitution, the strength of the gel network formed is the combined result of hydrophobic interactions and disulfide cross-links. Upon increasing the number of sulfhydryl groups, up to approximately six sulfhydryl groups for ovalbumin as tested under these conditions, the gel strength increases due to an increased possibility to undergo disulfide cross-links next to hydrophobic interactions. This was confirmed by the finding that these gels were poorly soluble in SDS while addition of β-mercaptoethanol lead to complete solubilization of the network (results not

shown). Upon increasing the degree of modification further, the gel strength decreases again due to an increasing repulsive force, induced by the concomitant introduction of carboxyl groups next to sulfhydryl groups, dominating the attractive forces in the absence of NaCl. In the presence of NaCl, the optimum shifts to the left with an optimum gel strength upon the introduction of four additional sulfhydryl groups. The evaluation of the gel hardness as shown in **Figure 6b** indicates clearly that four or six additional reactive sulfhydryl groups in the presence or absence of NaCl, respectively, lead to stronger gels. Increasing the degree of modification further resulted in a different and weaker network structure comprised of a denser network, as confirmed by the cryo-TEM micrographs (**Figure 4**) and rheological measurements (**Figures 6** and **7**). This is a combined effect of the reduced ability to form a hydrophobically reinforced network due to covalent linking and the development of a dense and differently organized gel network.

Conclusions. It was concluded that disulfide bond formation is preceded by physical interactions for ovalbumin aggregation and, as such, that disulfide interactions may not be the driving force for aggregation of ovalbumin. It was also found that the morphology of the formed aggregates is shifted from a fibrillar and linear aggregate system toward a highly branched and random aggregate system upon increasing the number of reactive sulfhydryl groups per protein. Even though the rate of aggregation is not affected by sulfhydryl groups, the morphology of the formed aggregates is clearly affected by their presence. These results suggest that even though aggregation of ovalbumin may be primarily driven by physical interactions, formed disulfide bonds can be of importance in determining the resulting aggregate morphology and rheological properties.

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