JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ovalbumin, Ovotransferrin, Lysozyme: Three Model Proteins for Structural Modifications at the Air−Water Interface

VALÉRIE LECHEVALIER,* THOMAS CROGUENNEC, STÉPHANE PEZENNEC, CATHERINE GUÉRIN-DUBIARD, MARYVONNE PASCO, AND FRANÇOISE NAU

UMR INRA-ENSAR Physico-Chimie et Technologie des Ovoproduits, CS 84215, 65 rue de Saint Brieuc, 35042 Rennes Cedex, France

Structural modifications of ovalbumin, ovotransferrin, and lysozyme at the air−water interface have been investigated using SDS-PAGE, both intrinsic and ANS fluorometry, and circular dichroism experiments. Ovalbumin contact with an interface induced an exposure of aromatic residues, a slight decrease in α-helix structures (−1.7%), and an increase in both β-sheet (+3.4%) and β-turn (+7.9%) structures. Moreover, these conformational changes led to the formation of insoluble polymers of ovalbumin through intermolecular disulfide bonds. Ovotransferrin contact with an interface led to an increase in its surface hydrophobicity (+30%) and modifications of its secondary structure (−33% of α-helices, +96.4% of β-sheets, +13.2% of β-turns, and +21.2% of random coils), characteristic of major conformational changes. On the other hand, lysozyme did not undergo any structural modification. These results clearly underscore that at the air−water interface proteins are susceptible to denaturation.

KEYWORDS: Ovalbumin; ovotransferrin; lysozyme; air−water interface; structure

INTRODUCTION

Egg white is traditionally used as an ingredient by many food industries because of its exceptional functional properties: foaming, emulsifying, and gelling properties. However, industrial egg white processing involves many technological treatments, such as mechanical treatments (stirring, blending, and pumping), thermal treatments (heating and cooling), as well as processing steps during which large interfacial areas are created, either with solid surfaces or with gas phases (transfer and spray-drying). These treatments impair the egg white functional properties (*1*−*3*). Mechanisms of protein diffusion to an air−water interface have been extensively studied (*4*−*9*). Moreover, several results demonstrate that proteins denature at an air−water interface (*10*−*15*). In some cases, intermolecular β-sheet formation has been demonstrated (*16*). The formation of intermolecular disulfide bonds between ovalbumin molecules after foaming has been highlighted (*17*). However, not so many studies have focused on the denaturation of hen egg white proteins at the air−water interface. They have, nevertheless, been shown to have different kinetics of adsorption (*9*), which suggests different levels of denaturation. The aim of the present study was to characterize the conformational changes of three major egg white proteins—ovalbumin, ovotransferrin, and lysozyme—at the air−water interface. These proteins were chosen for their physicochemical characteristics, which are representative of the diversity that can be found in egg white proteins. Ovalbumin, ovotransferrin, and lysozyme account for 54, 13, and 3.5% of the egg white proteins, respectively (*18*). Ovotransferrin is the biggest protein at 77.7 kDa, compared with 45 kDa for ovalbumin and 14.4 kDa for lysozyme. They also have different isoelectric points: ovalbumin is the more acid with a p*I* of 4.5, ovotransferrin is quasi neutral with a p*I* of 6.5, and lysozyme is very interesting because of its high isoelectric point (10.7), which makes it the only egg white protein that is positively charged in physiological conditions. Ovotransferrin is well-known for its iron binding capacity in relation with structural modifications of the protein (*19*). Ovalbumin is the only egg white protein with free sulfhydryl groups likely to enhance sulfhydryl−disulfide interchange reactions.

MATERIALS AND METHODS

Reagents. Ethylenediaminetetraacetic acid (EDTA), urea, acrylamide for fluorescence quenching analysis, L-tryptophan, anilino-1-naphthalene-8-sulfonate (ANS), and trichloroacetic acid (TCA) were purchased from Sigma Aldrich. Ellman's reagent [5′5-dithiobis(2-nitrobenzoic acid), DTNB], cesium chloride, and potassium iodide were obtained from Merck. β-Mercaptoethanol and acrylamide for electrophoresis analysis were purchased from Bio-Rad. Sodium dodecyl sulfate (SDS) was purchased from Biosolve Ltd.

Proteins. Ovalbumin and ovotransferrin were extracted from hen egg white by anion exchange chromatography according to the method of Croguennec et al. (*20*, *21*). Lysozyme was obtained from Ovonor (Annezin les Béthune, France).

Air—Water Interface Creation. Thirty milliliters of a 1% protein solution dissolved in 67 mM phosphate buffer, pH 7.0, was poured into a bubbling column PM 930 (Grosseron, St Herblain, France).

* Author to whom correspondence should be addressed [telephone (33) 2 23 48 55 75; fax (33) 2 23 48 55 78; e-mail lecheval@epi.roazhon.inra.fr].

10.1021/jf034184n CCC: $25.00 © 2003 American Chemical Society
Published on Web 09/06/2003

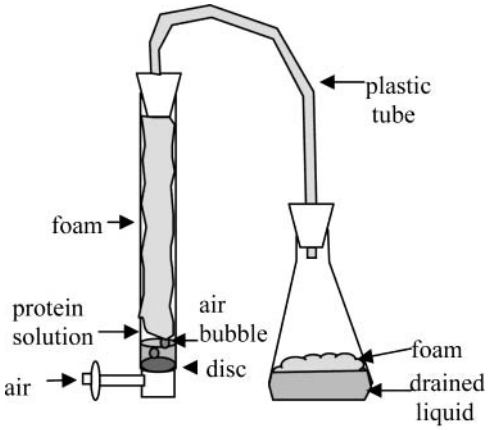

Figure 1. Scheme of the experimental material used for formation and recovery of the different samples.

Compressed air at a constant flow rate (75 mL min^{-1}) was injected through a metallic porous disk for 60 min. At the top of the column, foam was withdrawn via a plastic tube into a flask. This step lasted for <5 min, after which >95% of the protein solution was foamed (**Figure 1**). In the flask, drained liquid separated spontaneously from the foam during the remaining 55 min. This liquid was then reintroduced in the bubbling column. This sequence was repeated twice in order to obtain more foam, that is, to maximize the interface area. The drained liquid sample used for protein analysis was the last one, that is, obtained after three bubbling sequences. To minimize the liquid quantity still present in foam, a further centrifugation was applied (polypropylene tubes, 3000*g*, 10 min, 20 °C). For ovalbumin and ovotransferrin, the protein analysis indicated that the liquid obtained by centrifugation was equivalent to the drained liquid mentioned above; thus, results for the former sample will not be presented. Ovotransferrin foam was solubilized in 67 mM phosphate buffer, pH 7.0, whereas ovalbumin foam needed 10% SDS added to the same buffer to be solubilized. Foam from lysozyme was very unstable and collapsed during centrifugation, and then the whole solution obtained was considered to be the "foam" fraction.

Protein Concentration. Protein concentration in each sample was measured from the absorbance at 280 nm with ϵ_{280} = 32050, 91200, and 37750 cm^{-1} M^{-1} for ovalbumin, ovotransferrin, and lysozyme, respectively (*22*−*24*). Dilutions for sample preparation were based on these values.

Estimation of Protein Adsorbed at the Interface. The amount of protein adsorbed at the air−water interface was estimated as follows:

$$\text{adsorbed protein (\%)} = \frac{(C_{ns}V_{ns}) - (C_{dl}V_{dl} + C_sV_s)}{C_{ns}V_{ns}} \times 100 \quad (1)$$

C_{ns}, C_{dl}, and C_s are the protein concentration in native solution, drained liquid, and supernatant, respectively, and V_{ns}, V_{dl}, and V_s are the volumes of native solution, drained liquid, and supernatant, respectively.

Free and Total Sulfhydryl Groups. Free and total sulfhydryl groups were measured using Ellman's reagent (*25*). For quantification of the free sulfhydryl groups, 0.15 mL of sample solution was mixed with 2.85 mL of 0.1 M Tris-glycine buffer, pH 8.0, containing 0.01 M EDTA and 0.5% SDS. Then, 0.02 mL of DTNB solution was added. Absorbance was determined at 412 nm. For quantification of the total sulfhydryl groups, proteins were first reduced by adding 1 mL of 10 M urea and 0.02 mL of β-mercaptoethanol to the sample solutions. After 1 h in darkness and at room temperature, 10 mL of 12% TCA was added to the solution in order to precipitate out the proteins. Precipitated proteins were recovered by a 10 min centrifugation at 5000*g*. They were washed twice with 12% TCA to remove β-mercaptoethanol and then diluted in 3 mL of 0.1 M Tris-glycine buffer, pH 8.0, and 8 M urea. The subsequent steps were identical to those described for free sulfhydryl group quantification.

SDS-PAGE Analysis. SDS-PAGE was carried out according to the method of Laemmli (*26*), using a 7.5% acrylamide separating gel for ovalbumin and ovotransferrin, a 15% acrylamide separating gel for lysozyme, and a 4.5% acrylamide stacking gel, each containing 0.1% SDS. Protein samples were prepared in 0.15 M Tris-HCl buffer, pH 6.8, containing 2% SDS and 20% glycerol, with or without 0.5% β-mercaptoethanol. Electrophoresis was carried out at a constant current of 30 mA with an electrophoresis buffer of 0.15 M Tris-glycine, pH 8.6, containing 0.1% SDS. Gels were stained with Coomassie brilliant blue and destained in methanol/acetic acid/water (65:25:10 v/v/v) solution. Protein bands were quantified after being scanned, using Image Quant software.

Fluorescence Measurements. Intrinsic fluorescence measurements were performed using a spectrofluorometer LS50B (Perkin-Elmer). Proteins were diluted in 67 mM phosphate buffer, pH 7.0, in order to be in the linearity domain of fluorescence versus concentration. Protein solutions were excited at 280 or 295 nm, and emission spectra were registered between 305 and 415 nm with 1% attenuation. Excitation and emission slits were 15 nm. For each protein, three concentrations were tested to determine the slope of the relative fluorescence intensity versus protein concentration by linear regression analysis. The slope was then used as an index of the protein intrinsic fluorescence.

Quenching fluorescence measurements were performed with the spectrofluorometer LS50B described above. Two micromolar protein solutions were excited at 295 nm to minimize interference from tyrosyl residues. L-Tryptophan (L-Trp) solutions were used as the reference, with concentrations of $A \times 2$ μM, A being the number of tryptophyl residues in the protein (3 for ovalbumin, 11 for ovotransferrin, and 6 for lysozyme). Quenchers used were acrylamide (0−67 mM), cesium chloride (0−0.67 M), and potassium iodide (0−0.67 M). Potassium iodide solution contained 0.1 M sodium thiosulfate ($Na_2S_2O_3$) to prevent formation of I_3^-, which absorbs at 290 nm. With the quencher concentrations used, no shift of the maximal fluorescence intensity wavelength was observed, indicating the absence of protein denaturation. The quenching constant was expressed by the Stern−Volmer equation

$$F_0/F = 1 + K_q[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of the quencher, respectively, [Q] is the quencher concentration, and K_q is the quenching constant. The percentage of tryptophyl residues of the protein accessible to quenchers is calculated as follows:

$$\text{Trp \%} = [K_q(\text{sample})/K_q(\text{L-Trp})] \times 100 \quad (3)$$

Surface Hydrophobicity. Measurement of surface hydrophobicity was carried out using the fluorescence probe ANS. Proteins were diluted in the same way as for the intrinsic fluorescence measurement. Fifteen microliters of an 8 mM ANS solution was added to 1 mL of the protein sample solution. ANS fluorescence intensity was measured at 470 nm after excitation at 390 nm. Excitation and emission slits were 2.5, 10, and 15 nm for ovalbumin, ovotransferrin, and lysozyme solutions, respectively. The slope of the fluorescence intensity versus protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity (PSH), as suggested by Kato and Nakai (*27*). Finally, the relative surface hydrophobicity (RSH) was calculated as follows:

$$\text{RSH (\%)} = (\text{PSH of sample}) \times 100/(\text{PSH of control}) \quad (4)$$

The native solution sample was used as a control.

Circular Dichroism (CD). CD spectra were obtained using a CD 6 spectropolarimeter (Y. Jobin, Paris, France). Protein concentrations were 1.3, 0.77, and 0.34 g L^{-1} for ovalbumin, ovotransferrin, and lysozyme, respectively, to obtain absorbance at 280 nm between 0.8 and 1.0. Far-UV CD spectra were recorded from 180 to 250 nm with a 0.02 cm light path. Near-UV CD spectra were recorded from 250 to 330 nm with a 1 cm light path. Each spectrum was the average of three scans integrated with the data processor CD6DOS (Y. Jobin). CD spectra were expressed in terms of molar ellipticity [θ_λ]

$$[\theta_\lambda] = (3300\Delta A_\lambda)/(Cd) \quad (5)$$

with $[\theta_\lambda]$, the molar ellipticity at wavelength λ, expressed in deg cm^2 dmol^{-1}; ΔA_λ, the difference of absorbance of a right- and left-circular polarized light of equal intensity and of the same wavelength λ; C, the mean residue concentration when the far-UV CD spectrum was reported and the protein molarity in the near-UV region; and d (cm), the light path. α-Helix, β-sheet, β-turn, and random coil structures were determined from the far-UV CD spectra using CD spectra deconvolution software described by Bohm et al. (*28*).

Statistics. Data were analyzed using the statistical analysis package Statgraphics Plus, version 5.1. Intrinsic fluorescence, surface hydrophobicity, and CD data were analyzed using Student's t statistics.

RESULTS

To evaluate correctly the protein structural changes induced by foaming, the drained liquid and foam samples have always been compared to the native protein solutions in the corresponding buffer condition:

- For lysozyme and ovotransferrin, drained liquid and foam samples were compared to native lysozyme and ovotransferrin solutions in 67 mM phosphate buffer, pH 7.0, respectively.
- For ovalbumin, drained liquid samples were compared to the native ovalbumin solution in 67 mM phosphate buffer, pH 7.0; foam samples were compared to the native ovalbumin dissolved in 67 mM phosphate buffer, pH 7.0, added with 10% SDS, which must be used to solubilize ovalbumin foam. Thus, the differences observed were assigned to foaming.

Amount of Protein Adsorbed at the Air—Water Interface. As mentioned above, the foam behaviors depended on the protein. For lysozyme, foam completely collapsed with centrifugation. Then in eq 1, V_s was considered to be 0; the amount of lysozyme adsorbed at the interface (9.8 ± 4.2%) was consequently overestimated. For ovalbumin and ovotransferrin, 33.3% (±5.1%) and 20.2% (±4.2%) of the total proteins were adsorbed at the interface, respectively.

Protein Aggregation. Each fraction was compared to the corresponding native protein solution by SDS-PAGE under reducing and nonreducing conditions. In the presence of β-mercaptoethanol, no differences between the ovalbumin fractions were observed (**Figure 2A**). In the absence of β-mercaptoethanol (**Figure 2B**), a band at ∼80 kDa was observed for all fractions, and for the foam fraction, other bands at ∼116 and 170 kDa and more were also visible. The appearance of these high molecular weight bands occurred concomitantly with a decrease of the ovalbumin monomeric form quantity from 45% of the total protein amount in the native solution (**Figure 2B**, lane 3) to <30% in the foam fraction (**Figure 2B**, lane 4). Moreover, these values have to be compared to the 90% of ovalbumin monomeric form obtained when the foam fraction was analyzed by SDS-PAGE in the presence of β-mercaptoethanol (**Figure 2A**, lane 4). Ovalbumin aggregates present in the foam fraction would then involve disulfide bond interactions, probably resulting from sulfhydryl−disulfide interchange reactions during the exposure to the air−water interface. Free and total sulfhydryl measurements confirmed this hypothesis (**Figure 3**). The total number of sulfhydryl groups did not change between native ovalbumin molecules and ovalbumin molecules included in the foam fraction, but a 50% decrease in the number of free sulfhydryl groups was observed, suggesting that half of the ovalbumin free sulfhydryl groups were involved in disulfide bond formation.

For the ovotransferrin fractions, only one band at 77.7 kDa was visible on the SDS-PAGE in the presence (**Figure 2C**) and in the absence (**Figure 2D**) of β-mercaptoethanol. However, on the foam fraction lane and in the absence of reducing agent, this band was less intense, and some aggregates were present in the sample slot. This could explain the proportion of protein adsorbed at the interface previously mentioned. This result suggests that ovotransferrin became aggregated during foam formation probably through disulfide bond interactions, although there is no free sulfhydryl in native ovotransferrin.

For lysozyme analysis on SDS-PAGE in the presence (**Figure 2E**) and absence (**Figure 2F**) of β-mercaptoethanol, no differences between the fractions were observed. The only electrophoretic band was at 14.4 kDa, suggesting that no aggregation occurred during foam formation.

Fluorescence Spectra. The same fractions were analyzed by intrinsic fluorescence with the excitation wavelength at 280 and 295 nm, with and without quenchers. At 280 nm, both tyrosyl and tryptophyl residues are excited, whereas at 295 nm only tryptophyl residues are excited. At both excitation wavelengths, fluorescence emission spectra were registered between 305 and 415 nm.

For both excitation wavelengths, the emission spectra of ovalbumin fractions showed the same pattern (data not shown). No variations in the fluorescence intensity of drained liquid solution were observed, but we noticed a significant red shift of 0.4 nm (Student's t test, $P \leq 0.05$) using a λ_{ex} at 280 nm. The red shift was not statistically significant using a λ_{ex} at 295 nm. This result would indicate an exposure of tyrosyl residues. On the other hand, a drastic decrease of the fluorescence intensity (−61% at 280 nm and −70% at 295 nm) was observed for the foam fraction, compared to the native ovalbumin solution with SDS (**Figure 4**). This suggested significant structural modifications due to foaming. Moreover, native ovalbumin solution in the presence of SDS showed a 29% lower fluorescence intensity using an excitation at 295 nm, compared with that at 280 nm, whereas a 46% loss was observed for the foam fraction (**Figure 4**). Thus, tyrosyl residues seemed to contribute more to the fluorescence of the foam fraction than to that of the native solution. Moreover, between the native protein and the foam fraction, the maximal emission wavelength was shifted to the longer wavelengths, which would suggest the exposure of the aromatic residues to a less hydrophobic environment (**Figure 5**). Significant maximum emission intensity red shifts of 0.7 nm (Student's t test, $P \leq 0.001$) and 0.5 nm (Student's t test, $P \leq 0.05$) were obtained when excitation wavelengths of 280 and 295 nm were used, respectively, confirming the important participation of tyrosyl residues in fluorescence emission. Fluorescence quenching data indicated that tryptophyl residues were also exposed to a less hydrophobic environment in the foam fraction (**Table 1**), indicating structural modifications: accessibility of tryptophyl residues to acrylamide, cesium, and iodide was increased by 100% between the native solution in the presence of SDS and the foam fraction.

The results for ovotransferrin solution were quite similar to those obtained with ovalbumin. Fluorescence emission intensity was higher at 280 nm than at 295 nm. With an excitation wavelength at 280 nm, the fluorescence intensity was 49% higher for the native ovotransferrin solution and 59% higher for the foam fraction (**Figure 4**). Significant red shifts of 0.8 nm (Student's t test, $P \leq 0.001$) for λ_{ex} at 280 nm and of 0.6 nm (Student's t test, $P \leq 0.05$) for λ_{ex} at 295 nm were also observed (**Figure 5**). Thus, tyrosyl residues appeared to be more affected by the structural modifications due to contact with the air−water interface. For the foam fraction, fluorescence quenching data showed a 100% increase in the number of tryptophyl residues quenched by acrylamide and a lower increase (25%)

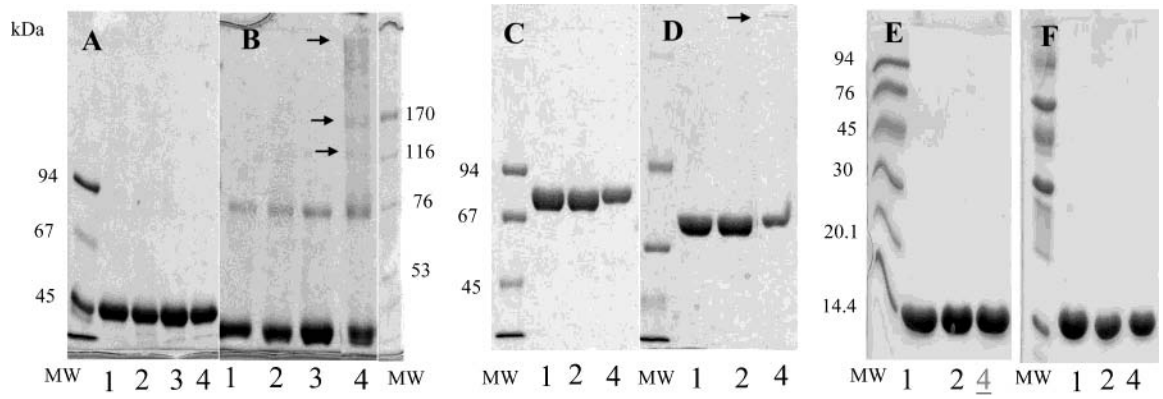

Figure 2. SDS-PAGE, with (A, C, E) and without (B, D, F) mercaptoethanol, of ovalbumin solutions (A, B), ovotransferrin solutions (C, D), and lysozyme solutions (E, F): (lane 1) native solution; (lane 2) drained liquid; (lane 3) native solution + 10% SDS; (lane 4) foam; (MW) molecular weight.

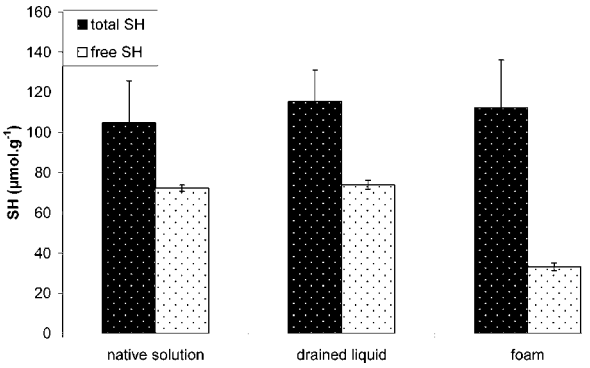

Figure 3. Ovalbumin total and free sulfhydryl group measurement in native solution, drained liquid, and foam fraction.

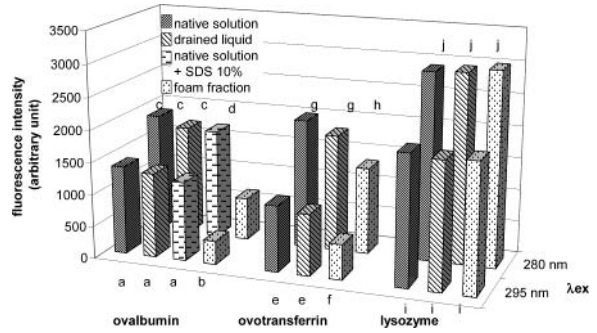

Figure 4. Evolution of the maximum intrinsic fluorescence intensity after excitation at 280 or 295 nm for ovalbumin, ovotransferrin, and lysozyme. Samples with different letters are statistically different ($P \leq 0.05$).

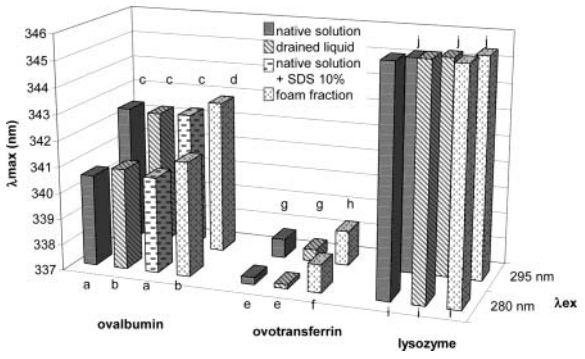

Figure 5. Evolution of maximal emission wavelength in intrinsic fluorescence after excitation at 280 or 295 nm for ovalbumin, ovotransferrin and lysozyme. Samples with different letters are statistically different ($P \leq 0.05$).

in those quenched by iodide. On the other hand, no tryptophyl residue was accessible to cesium as deduced from the nonsignificant decrease of fluorescence intensity after cesium addition (**Table 1**). For drained liquid, no shifts in emission maximum intensity and no variations of intensity were observed.

Lysozyme showed the same fluorescence intensity decrease between λ_{ex} at 280 nm and λ_{ex} at 295 nm for both the native solution and the foam fraction (−35%) (**Figure 4**) and with no shifts (**Figure 5**). No significant increase in the number of tryptophyl residues accessible to the quenchers (**Table 1**) was observed ($P \leq 0.05$). Accessibility of tryptophyl residues to cesium and iodide was equivalent for the three fractions (native solution, drained liquid, and foam). Concerning accessibility to acrylamide, despite different mean values, the standard deviation did not permit a conclusion to be drawn.

Surface Hydrophobicity. The surface hydrophobicities of proteins in the drained liquid and foam fractions were compared to the surface hydrophobicity of native protein (before foam formation). Ovalbumin molecules in native solution and drained liquid showed the same surface hydrophobicity (**Figure 6**). Addition of 10% SDS was necessary to solubilize the ovalbumin foam. For the native solution, this surfactant caused an important increase in surface hydrophobicity (Student's t test, $P \leq 0.001$), indicating major structural modifications. Subsequently, the surface hydrophobicity of ovalbumin molecules included in the foam fraction was compared to that of native proteins in the presence of 10% SDS. The increase was of the order of 100% for the foam fraction, indicating that ovalbumin molecules present in foam behaved differently from those of the native solution in the presence of SDS. This probably implies that ovalbumin molecules in foam are denatured by contact with the air−water interface.

In the ovotransferrin foam fraction, a significant increase in ovotransferrin molecule surface hydrophobicity was observed for the foam fraction (+30%, $P \leq 0.05$), compared to native ovotransferrin molecules. No significant change was observed for the drained liquid.

Lysozyme exposure to the air−water interface led to a significant increase in its surface hydrophobicity (+21.5%, $P \leq 0.05$), whereas the surface hydrophobicity of lysozyme molecules in drained liquid was not statistically different from native lysozyme surface hydrophobicity.

Circular Dichroism Spectra. Any change in the environment of aromatic residues and disulfide bonds results in a change of the near-UV CD spectra. No significant difference was observed between the ovalbumin native solution and drained liquid (**Figure 7A**). The presence of SDS in the native solution slightly modified the spectrum, especially between 250 and 260 nm

Table 1. Percentage of Tryptophyl Residues Accessible to Different Quenchers for Each Protein[a]

	acrylamide			cesium			iodide		
	ova	ovtf	lyso	ova	ovtf	lyso	ova	ovtf	lyso
native solution	27.9a ± 1.3	11.2a ± 3.8	59.1a ± 8.1	9.8a ± 0.6	3.9a ± 3.9	27.4a ± 1.3	5.1a ± 0.1	4.7a ± 0.2	39.7a ± 0.6
drained liquid	26.8a ± 1.7	11.7a ± 1.7	32.3a ± 2.5	10.6a ± 0.7	1.9a ± 1.9	33.1a ± 2.7	4.3a ± 0.4	5.1a ± 0.3	39.5a ± 0.7
native solution + SDS	20.2b ± 0.6	nd	nd	12.9a ± 1.6	nd	nd	5.6a ± 0.02	nd	nd
foam fraction	35 c ± 3.4	21.7b ± 2.1	49.9a ± 8.3	31.7b ± 4.5	0.4a ± 0.3	34 a ± 6.7	12.6b ± 1.7	6.5b ± 0.5	35.8a ± 1.1

[a] Results with different letters are statistically different (Student's *t* test, $P \leq 0.05$); nd, not determined.

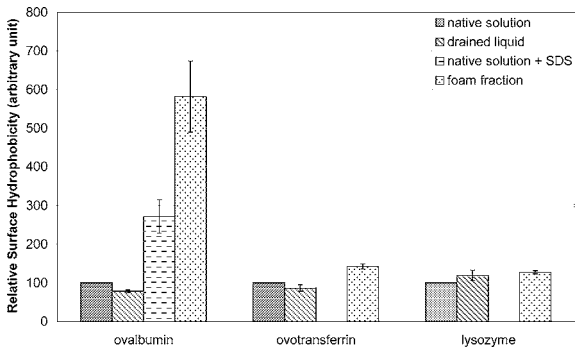

Figure 6. Evolution of surface hydrophobicity for ovalbumin, ovotransferrin, and lysozyme.

(absorption range of disulfide bonds) and around 280 nm (absorption range of aromatic residues). However, these variations were not statistically significant. The foam fraction CD spectrum was significantly different from the CD spectra of other ovalbumin fractions.

For ovotransferrin, the CD spectra of the native solution and drained liquid were not statistically different (**Figure 7B**). In comparison, the foam fraction CD spectrum was completely modified, suggesting that the tertiary structure of ovotransferrin was different after contact with the air−water interface.

For lysozyme, the CD spectra of the native solution, drained liquid, and foam fraction were not statistically different (**Figure 7C**), assuming an absence of any modification of its tertiary structure.

Far-UV CD spectra enabled us to evaluate the protein secondary structure (**Table 2**). However, it has to be pointed out that the deconvolution method used influences the proportions of protein secondary structure. This explains why some of the present results did not exactly fit with the literature data. For example, the α-helix proportion determined for ovalbumin (∼18%) was weaker than the one given by Stein et al. (*29*) (∼30%), and the β-sheet proportion determined for lysozyme (∼20%) was higher than the one given by Harata (*30*) (∼6%). Nevertheless, only the relative variations, revealing structural modifications and independent of the deconvolution software used, have been considered in the following comments. For ovalbumin, the presence of 10% SDS in the native solution did not significantly change the spectrum (**Figure 8A**). Drained liquid and foam fraction spectra were significantly different from the original solution spectrum ($P \leq 0.05$). Thus, after exposure to the air−water interface, ovalbumin underwent conformational changes implying secondary structure modifications. α-Helix structure losses of 1.8 and 1.7%, for drained liquid and foam fraction, respectively ($P \leq 0.05$), were calculated with the CDNN spectra deconvolution software. For drained liquid, a 4.7% decrease of β-sheet structures was observed, whereas for the foam fraction β-sheet structures increased by 3.4%. A decrease of β-turn structures by 15.7% was observed for drained liquid, whereas for the foam fraction this increased by 7.9%. Random coil structures were unmodified for the foam fraction, but an increase of 18.3% for the drained liquid was observed.

The far-UV CD spectra of ovotransferrin native solution and drained liquid were not statistically different (**Figure 8B**). However, the foam fraction CD spectrum was very different, showing a large modification of its secondary structure. A significant loss of α-helix structures (33%) and gains of β-sheet (96.4%), β-turn (13.2%), and random coil (21.2%) structures were observed ($P \leq 0.01$).

For lysozyme, no significant difference was observed between the far-UV CD spectra of the native solution, drained liquid, and foam fraction (**Figure 8C**).

DISCUSSION

The present results demonstrate that the exposure of ovalbumin to the air−water interface generates its aggregation, through intermolecular disulfide bonds. Kitabatake and Doi (*17*) have already made such observations, assuming the unfolding of the molecule following contact with the air−water interface. Such an aggregation has also been observed by Kato et al. (*31*) and Mine et al. (*32*) after heating egg white to >60 °C. Linear aggregates were also obtained after ovalbumin had been heated to >80 °C (*33*). These aggregation phenomena imply the exposure of sulfhydryl groups, which means changes in the tertiary structure of ovalbumin. The present results of intrinsic fluorescence, surface hydrophobicity, and near-UV CD agree with this assumption.

After contact with the air−water interface, tyrosyl residues take part more strongly in ovalbumin fluorescence, as indicated by the ratio of fluorescence intensity after excitation at 280 nm to that after excitation at 295 nm. The red shift was also more significant after excitation at 280 nm. Thus, the contact with the interface results predominantly in the exposure of tyrosyl residues. The red shift observed for drained liquid at 280 nm, but absent at 295 nm, also suggests that tyrosyl residues are the first to be exposed. The exposure of tryptophyl residues is suggested by their increasing accessibility to fluorescence quenchers. Such exposure of aromatic residues involves modifications in the tertiary structure of ovalbumin. It is noticeable that the extent of these changes (fluorescence intensity decrease of 43% with a red shift) seems to be higher than that induced by heating at 80 °C (fluorescence intensity decrease of 56% without red shift) but lower than that resulting from the addition of guanidium hydrochloride (fluorescence intensity decrease of 64% with a red shift) (*34*).

Structural modifications of proteins are classically demonstrated through surface hydrophobicity measurements, using specific probes such as ANS. However, these methods imply that the proteins are soluble and, after contact with the air−water interface (foam fraction), ovalbumin needs SDS to be solubilized. Unfortunately, SDS increases the surface hydrophobicity measurement for ovalbumin, indicating significant structural changes due to SDS, as mentioned by Hayakawa et

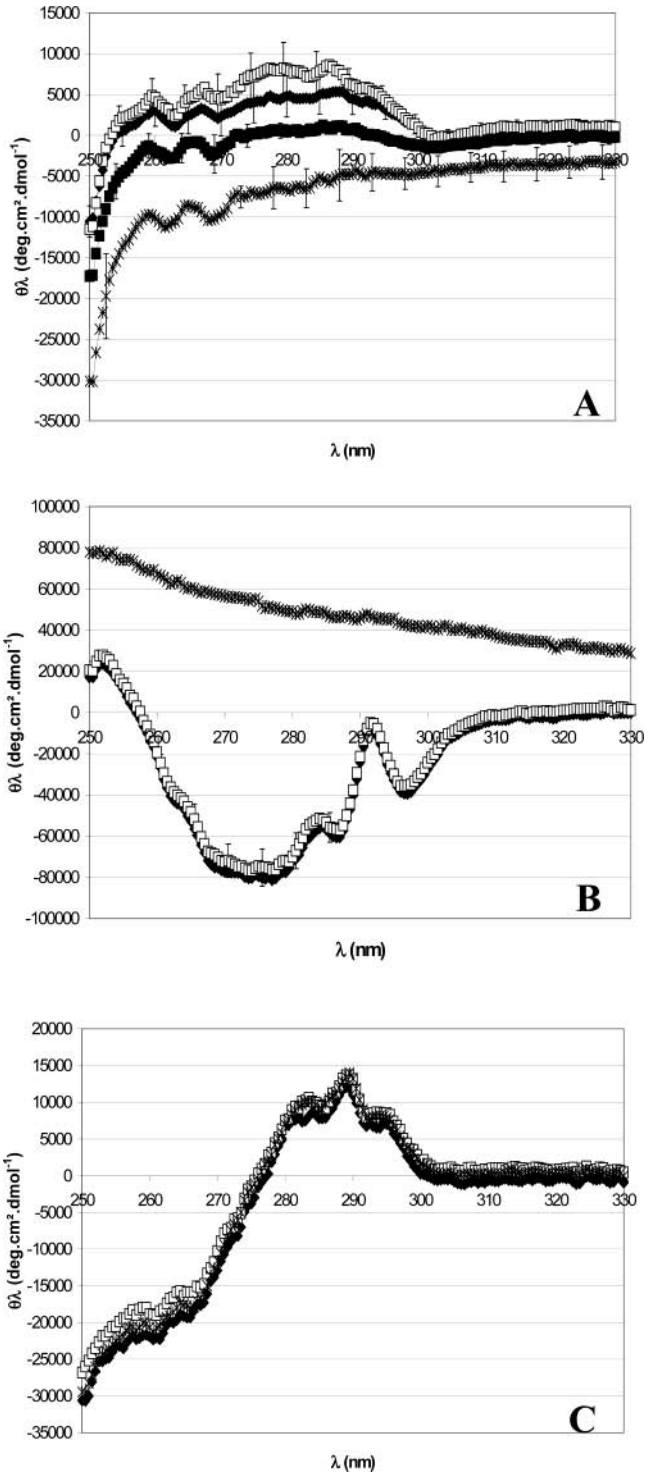

Figure 7. Near-UV CD spectra of ovalbumin (A), ovotransferrin (B), and lysozyme (C): (◆) native solution; (□) drained liquid; (■) native solution + SDS; (∗) foam fraction.

al. (*35*). However, a significant difference was observed between native ovalbumin in the presence of SDS and the foam fraction. This result reinforces the assumption of tertiary structure modifications for ovalbumin after contact with the air−water interface. Structural modifications agree with the red shift observed in intrinsic fluorescence, assuming the unfolding of the molecule. Moreover, the increase in surface hydrophobicity after contact with the air−water interface is similar to those obtained from mild heat treatment of ovalbumin (*7*, *31*, *32*, *34*). These results, that is, nearly no secondary structural changes

Table 2. Percentage of Secondary Structure for Each Protein Calculated from Far-UV CD Spectra Using CDNN Spectra Deconvolution Software (*28*)

	α-helix	β-sheet	β-turn
ovalbumin			
native	17.8 ± 0.1	36.9 ± 0.3	19.2 ± 2.4
drained liquid	17.5 ± 0.1	35.2 ± 0.6	16.2 ± 0.2
native + SDS 10%	17.7 ± 0.2	37.4 ± 0.5	19.6 ± 1.9
foam + SDS 10%	17.4 ± 0.1	38.7 ± 0.5	21.2 ± 0.0
ovotransferrin			
native	28.3 ± 0.05	23.4 ± 0.1	18.1 ± 0.0
drained liquid	28.1 ± 0.05	23.6 ± 0.05	18.1 ± 0.0
foam	19.0 ± 0.9	45.9 ± 3.4	20.5 ± 0.4
lysozyme			
native	29.9 ± 0.7	22.3 ± 1.3	18.2 ± 0.3
drained liquid	31.5 ± 0.3	20.4 ± 0.3	18.0 ± 0.1
foam	30.7 ± 0.5	21.3 ± 0.7	18.1 ± 0.1

but tertiary structure modifications and increasing binding of a hydrophobic fluorescence dye, would suggest that after contact with the interface, ovalbumin would evolve into a "molten-globule-like" state as defined by Tani et al. (*34*).

The results of near-UV CD confirmed the changes of ovalbumin tertiary structure around the aromatic residues. Koseki et al. (*36*) had already mentioned such changes, reversible after heating ovalbumin to below 65 °C and irreversible above 65 °C. Moreover, Tani et al. (*34*) demonstrated tertiary structure modifications after heat treatment. Exposure of ovalbumin at the air−water interface induced tertiary structure modifications that seem close to those observed after heat treatment of this protein.

Concerning the secondary structure, after contact with the air−water interface, the variations observed were 10-fold lower than those observed after heat denaturation of ovalbumin (*5*, *32*, *37*). However, in both cases, there was a loss of α-helix and a gain of β-sheet, β-turn, and random coil structures. The slight increase in β-sheets could be explained by the appearance of intermolecular β-sheets, as suggested by Renault et al. (*16*).

Ovotransferrin differs from ovalbumin by its size, its conformation, its number of disulfide bridges, and, above all, the absence of free sulfhydryl groups. Considering this fact, the absence of aggregation through sulfhydryl−disulfide interchanges revealed by the SDS-PAGE analysis was foreseeable. However, the aggregates observed in the sample slot in the absence of β-mercaptoethanol were more unexpected. Contamination by a very small quantity of ovalbumin could probably be responsible for such an aggregation, the reduction of ovotransferrin intramolecular disulfide bonds being initiated by the ovalbumin free sulfhydryl groups and enabling ovotransferrin intermolecular disulfide bonds. This assumption agrees with the observations of Xu et al. (*38*) and Watanabe et al. (*39*): disulfide bonds between ovotransferrin molecules appeared during heat treatment in the presence of ovalbumin. Moreover, and as mentioned above, ovalbumin acquires an appropriate conformation to initiate this kind of reaction after contact with the air−water interface.

However, these results suppose, as well, that ovotransferrin would also be in a more reactive conformation. Actually, the aromatic residues are partially exposed after contact with the air−water interface, as indicated by the increase in surface hydrophobicity, the red shift observed, and the increasing accessibility of tryptophyl residues to acrylamide. Also, the drastic modifications of the near-UV CD spectrum highlight the major changes in the tertiary structure of ovotransferrin.

It is noticeable that the three quenchers used cannot reach the tryptophyl residues in the same way: cesium and iodide,

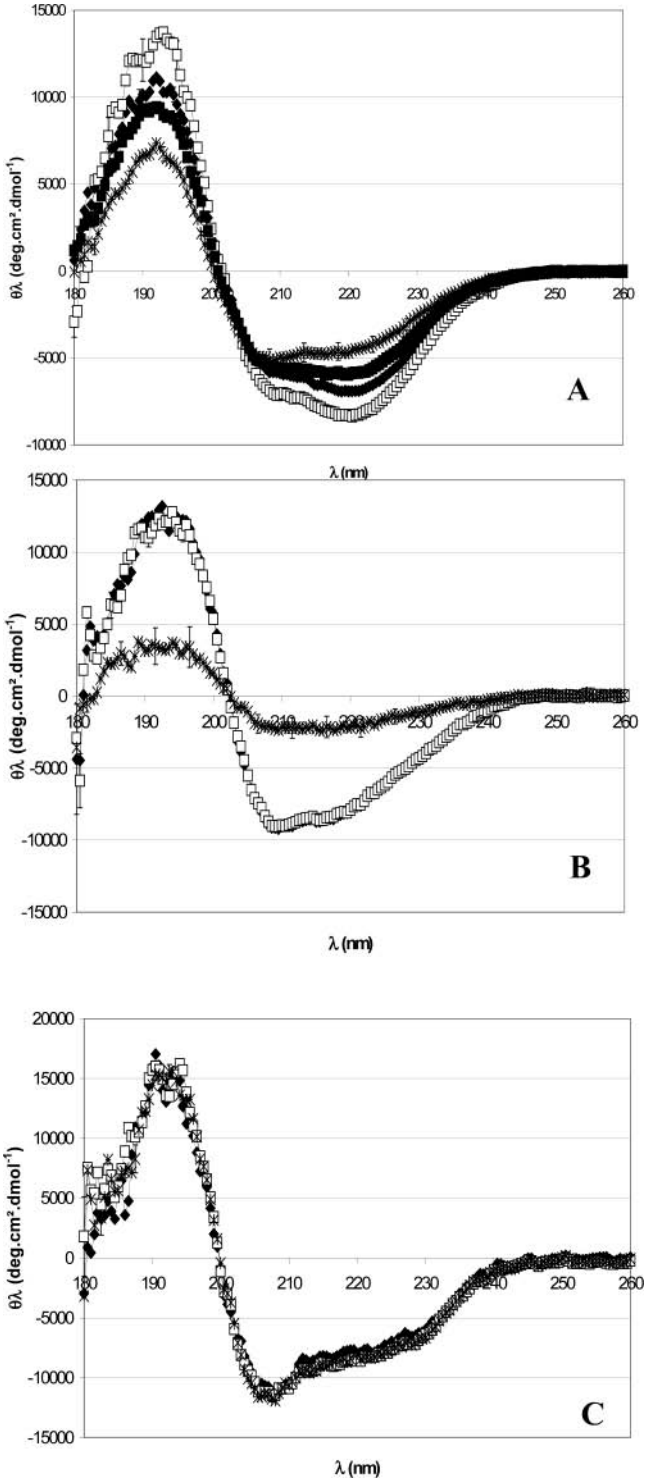

Figure 8. Far-UV CD spectra of ovalbumin (A), ovotransferrin (B), and lysozyme (C): (◆) native solution; (□) drained liquid; (■) native solution + SDS; (∗) foam fraction.

which are charged ions, appear to be repelled from ovotransferrin molecules. This is probably due to the charges of the amino acid side chains surrounding the tryptophyl residues. Actually, the primary sequence of ovotransferrin shows many charged amino acids besides the tryptophyl residues. Most of them are positively charged (Arg, Lys, and His), which agrees with the complete rejection of cesium cations, but some tryptophyl residues are near negatively charged residues (Glu and Asp), explaining their low accessibility to iodide anions.

The secondary structure also appeared to be greatly altered, corresponding to significant denaturation with, in particular, a strong decrease in α-helices (−33%) and a high increase in *β*-sheets (almost a 100% increase).

Structural modifications of ovotransferrin molecules adsorbed in the foam fraction are not observed for the drained liquid fraction. This suggests that conformational changes undergone by ovotransferrin at the air−water interface were so significant that the protein could not desorb any more.

Lysozyme is a small compact molecule with four disulfide bonds that make it very rigid. This conformation is probably responsible for the quasi-absence of denaturation after exposure to the air−water interface. Actually, no changes are observed concerning the aromatic residues, as opposed to what was observed after heat treatment at 80 °C or addition of guanidium chloride (*34*). Moreover, no modifications of CD spectra have been obtained after contact with the interface, in contrast to structural modifications induced by heat or chemical treatments (*34*, *40*). The only noticeable change was a 21% increase in the surface hydrophobicity, whereas a 93% increase was observed after thermal denaturation (*27*, *34*).

Finally, the behavior of lysozyme at the air−water interface seems very close to *β*-lactoglobulin behavior. For this protein, Phillips et al. (*41*) talked about a 25% increase in the surface hydrophobicity. These similarities are probably connected to the structural resemblance between lysozyme and *β*-lactoglobulin, both of which are small proteins (14.4 and 18.3 kDa, respectively) with four disulfide bonds.

Kato et al. (*5*) provided evidence of the correlation between the increase in surface hydrophobicity and the loss of α-helix structures for lysozyme and ovalbumin. The present study shows that for lysozyme that is in contact with an air−water interface, the slight increase in surface hydrophobicity was observed independently of any modification of secondary structure.

Faced with denaturation at the air−water interface, ovalbumin, ovotransferrin, and lysozyme, which are three major egg white proteins, exhibit very different behaviors. Lysozyme is unaffected by such a treatment, whereas ovotransferrin undergoes dramatic modifications concerning secondary structure as well as tertiary structure. Among these three proteins, ovotransferrin is clearly the more significantly denatured, in agreement with its already described flexible nature (*19*), highly sensitive to any denaturation.

Ovalbumin undergoes secondary and tertiary structure modifications that are weaker than those for ovotransferrin. However, because of its free sulfhydryl groups, these changes are enough to enable intermolecular sulfhydryl−disulfide exchanges, leading to aggregation and polymerization of ovalbumin at the air−water interface. This specific behavior is responsible for the very stable foam.

ACKNOWLEDGMENT

We are grateful to Pr. G. Brulé for helpful discussions, M. Piot for technical assistance, and V. James for English corrections. We gratefully acknowledge D. Marion for supplying CD facilities.

LITERATURE CITED

(1) Britten, M.; Lavoie, L. Foaming properties of proteins as affected by concentration. *J. Food Sci.* **1992**, *57*, 1219−1222.
(2) Matringe, E.; Phan Tan Luu, R.; Lorient, D. Functional properties of milk-egg mixtures. *J. Food Sci.* **1999**, *64*, 787−791.

(3) Croguennec, T.; Nau, F.; Brulé, G. Influence of pH and salts on egg white gelation. *J. Food Sci.* **2002**, *67*, 608−614.
(4) Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. I—Kinetics of adsorption and surface denaturation. *J. Colloid Interface Sci.* **1979**, *70*, 403−414.
(5) Kato, A.; Tsutsui, N.; Matsudomi, N.; Kobayashi, K.; Nakai, S. Effects of partial denaturation on surface properties of ovalbumin and lysozyme. *Agric. Biol. Chem.* **1981**, *45*, 2755−2760.
(6) De Feijter, J. A.; Benjamins, J. Adsorption kinetics of protein at the air−water interface. In *Food Emulsions and Foams*; Dickinson, E., Stainsby, G., Eds.; Royal Society of Chemistry: London, U.K., 1987; pp 285−330.
(7) Kitabatake, N.; Indo, K.; Doi, E. Changes in interfacial properties of hen egg ovalbumin caused by freeze-drying and spray-drying. *J. Agric. Food Chem.* **1989**, *37*, 905−910.
(8) Xu, S.; Damodaran, S. Mechanism of adsorption of lysozyme at the air−water interface. In *Food Proteins—Structure and Functionality*; Schwenke, K. D., Mothes, R., Eds.; VCH: Weinheim, Germany, 1993; pp 270−277.
(9) Damodaran, S.; Anand, K.; Razumovsky, L. Competitive adsorption of egg white proteins at the air−water interface: direct evidence for electrostatic complex formation between lysozyme and other egg proteins at the interface. *J. Agric. Food Chem.* **1998**, *46*, 872−876.
(10) Ter-Minassian-Saraga, L. Protein denaturation on adsorption and water activity at interfaces: an analysis and suggestion. *J. Colloid Interface Sci.* **1981**, *80*, 393−401.
(11) Song, K. B.; Damodaran, S. Structure−function relationship of proteins: adsorption of structural intermediates of bovine serum albumin at the air−water interface. *J. Agric. Food Chem.* **1987**, *35*, 236−241.
(12) Damodaran, S. Interfaces, protein films, and foams. *Adv. Food Nutr. Res.* **1990**, *34*, 1−79.
(13) Tronin, A.; Dubrovsky, T.; Dubrovskaya, S.; Radicchi, G.; Nicolini, C. Role of protein unfolding in monolayer formation on air−water interface. *Langmuir* **1996**, *12*, 3272−3275.
(14) Clarkson, J. R.; Cui, Z. F.; Darton, R. C. Protein denaturation in foam. II—Surface activity and conformational change. *J. Colloid Interface Sci.* **1999**, *215*, 333−338.
(15) Meinders, M. B. J.; de Jongh H. H. J. Limited conformational change of β-lactoglobulin upon adsorption at the air−water interface. *Biopolymers* **2002**, *67*, 319−322.
(16) Renault, A.; Pezennec, S.; Gauthier, F.; Vié, V.; Desbat, B. The surface rheological properties of native and S-ovalbumin are correlated with the development of an intermolecular β-sheet network at the air−water interface. *Langmuir* **2002**, *18*, 6887−6895.
(17) Kitabatake, N.; Doi, E. Conformational change of hen egg ovalbumin during foam formation detected by 5,5′-dithiobis(2-nitrobenzoic acid). *J. Agric. Food Chem.* **1987**, *35*, 953−957.
(18) Stadelman, W. J.; Cotterill, O. J. *Egg Science and Technology*, 2nd ed.; Avi Publishing: Westport, CT, 1977.
(19) Jeffrey, P. D.; Bewley, M. C.; MacGillivray, R. T. A.; Mason, A. B.; Woodworth, R. C.; Baker, E. N. Ligand-induced conformational change in transferrins: crystal structure of the open form of the N-terminal half molecule of human transferrin. *Biochemistry* **1998**, *37*, 13978−13986.
(20) Croguennec, T.; Nau, F.; Pezennec, S.; Brulé, G. Simple rapid procedure for preparation of large quantity of ovalbumin. *J. Agric. Food Chem.* **2000**, *48*, 4883−4889.
(21) Croguennec, T.; Nau, F.; Pezennec, S.; Piot, M.; Brulé, G. Two-step chromatographic procedure for the preparation of hen egg white ovotransferrin. *Eur. Food Res. Technol.* **2001**, *212*, 296−301.
(22) Hatta, H.; Kitabatake, N., Doi, E. Turbidity and hardness of a heat-induced gel of hen egg ovalbumin. *Agric. Biol. Chem.* **1986**, *50*, 2083−2089.
(23) Lin, L. N.; Mason, A. B.; Woodworth, R. C.; Brandts, J. F. Calorimetric studies of the binding of ferric ions to ovotransferrin and interactions between binding sites. *Biochemistry* **1991**, *30*, 11660−11669.
(24) Aune, K.; Tanford, C. Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. I. Dependence on pH at 25 °C. *Biochemistry* **1969**, *8*, 4579.
(25) Beveridge, T.; Toma, S. J.; Nakai, S. Determination of SH- and SS- groups in some food proteins using Ellman's reagent. *J. Food Sci.* **1974**, *39*, 49.
(26) Laemmli, U. K. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(27) Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.
(28) Bohm, G.; Muhr, R.; Jaenicke, R. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng.* **1992**, *5*, 191−195.
(29) Stein, P. E.; Leslie, A. G. W.; Finch, J. T.; Carrell, R. W. Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. *J. Mol. Biol.* **1991**, *221*, 941−959.
(30) Harata, K. X-ray structure of monoclinic form of hen egg white lysozyme crystallized at 313 K: comparison of two independent molecules. *Acta Crystallogr. Biol. Crystallogr.* **1994**, *50*, 250.
(31) Kato, A.; Matsuda, T.; Matsudomi, N.; Kobayashi, K. Difference in the molecular weight of soluble aggregates during heat denaturation between ovalbumin and S-ovalbumin. *Agric. Biol. Chem.* **1985**, *49*, 2197−2198.
(32) Mine, Y.; Noutomi, T.; Haga N. Thermally induced changes in egg white proteins. *J. Agric. Food Chem.* **1990**, *38*, 2122−2125.
(33) Koseki, T.; Fukuda, T.; Kitabatake, N.; Doi, E. Characterization of linear polymers induced by thermal denaturation of ovalbumin. *Food Hydrocolloids* **1989**, *3*, 135−148.
(34) Tani, F.; Murata, M.; Higasa, T.; Goto, M.; Kitabatake, N.; Doi, E. Molten globule state of protein molecules in heat-induced transparent food gels. *J. Agric. Food Chem.* **1995**, *43*, 2325−2331.
(35) Hayakawa, I.; Kajihara, J.; Morikawa, K.; Oda, M.; Fujio, Y. Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. *J. Food Sci.* **1992**, *57*, 288−292.
(36) Koseki, T.; Kitabatake, N.; Doi, E. Irreversible thermal denaturation and formation of linear aggregate of ovalbumin. *Food Hydrocolloids* **1989**, *3*, 123−134.
(37) Kato, A.; Takagi, T. Formation of intermolecular β-sheet structure during heat denaturation of ovalbumin. *J. Agric. Food Chem.* **1988**, *36*, 1156−1159.
(38) Xu, J. Q.; Shimoyamada, M.; Watanabe, K. Heat aggregation of dry-heated egg white and its inhibiting effect on heat coagulation of fresh egg white. *J. Agric. Food Chem.* **1998**, *46*, 3027−3032.
(39) Watanabe, K.; Nakamura, Y.; Xu, J. Q.; Shimoyamada, M. Inhibition against heat coagulation of ovotransferrin by ovalbumin dry heated at 120 °C. *J. Agric. Food Chem.* **2000**, *48*, 3965−3972.
(40) Nohara, D.; Mizutani, A.; Sakai, T. Kinetic study on thermal denaturation of hen egg white lysozyme involving precipitation. *J. Biosci. Bioeng.* **1999**, *87* (2), 199−205.
(41) Phillips, L. G.; Hawks, S. E.; German, J. B. Structural characteristics and foaming properties of β-lactoglobulin: effect of shear rate and temperature. *J. Agric. Food Chem.* **1995**, *43*, 613−619.

Received for review February 24, 2003. Revised manuscript received July 17, 2003. Accepted August 8, 2003. This work was supported by the Conseil Régional de Bretagne and the Conseil Régional des Pays de la Loire.

JF034184N