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Food Chemistry 92 (2005) 79–87
Food Chemistry
www.elsevier.com/locate/foodchem

Evidence for synergy in the denaturation at the air–water interface of ovalbumin, ovotransferrin and lysozyme in ternary mixture

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Received 5 April 2004; received in revised form 20 July 2004; accepted 20 July 2004

Abstract

The conformational changes of egg-white proteins, in a ternary-protein system, at the air–water interface have been studied. Three of the major egg-white proteins, ovalbumin, ovotransferrin and lysozyme, were studied with concentration ratios reflecting those in egg-white. Results were compared to those obtained in a previous work on protein denaturation at the air–water interface in single-protein systems (Lechevalier, V., Croguennec, T., Pezennec, S., Guérin-Dubiard, C., Pasco, M., & Nau, F. (2003). Ovalbumin, ovotransferrin, lysozyme: Three model proteins for structural modifications at the air–water interface. *Journal of Agricultural and Food Chemistry 51*, 6354–6361). Foaming altered the protein structure more profoundly in the mixture than in single-protein systems. Strong electrostatic interactions were observed between the three proteins. Their existence at the air–water interface could ease intermolecular sulfhydryl–disulfide exchange reactions between ovalbumin and both ovotransferrin and lysozyme. This study highlighted the fact that results obtained on single-protein systems were not easily extrapolable to complex systems, such as egg-white.

Keywords: Ovalbumin; Ovotransferrin; Lysozyme; Air–water interface; Protein mixture; Structure

1. Introduction

Egg albumen is extensively used as a functional ingredient in processed foods because of its excellent interfacial properties. Several studies have been reported on the respective roles of the different proteins in the expression of egg albumen surface active properties (Acton, Kropp, & Dick, 1990; Johnson & Zabik, 1981; Li-Chan & Nakai, 1989). Particularly, many authors have described adsorption kinetics of egg-white proteins at the air–water interface (Damodaran, Anand, & Razumovsky, 1998; De Feijter & Benjamins, 1987; Hunter, Kilpatrick, & Carbonell, 1990; Xu & Damodaran, 1993).

Many other works have been carried out to study protein structural changes at the air–water interface, but mainly on single-protein systems (Kitabatake & Doi, 1987; Lechevalier et al., 2003; Renault, Pezennec, Gauthier, Vié, & Desbat, 2002). In a previous work (Lechevalier et al., 2003), differences of behaviour at the air–water interface, of three major egg white proteins, namely, ovalbumin, ovotransferrin and lysozyme, in single-protein systems were described. Although, lysozyme did not suffer any structural modification at the air–water interface, ovalbumin and ovotransferrin underwent large rearrangements. Ovalbumin unfolding led to the formation of insoluble polymers, mostly through intermolecular disulfide bonds. Ovotransferrin unfolded at the air–water interface with the disappearance of almost all secondary and tertiary structures.

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doi:10.1016/j.foodchem.2004.07.006

However, egg-white is a complex mixture of proteins, and many competitions and interactions between proteins at the air–water interface can occur. Some authors have highlighted such phenomena, especially Damodaran et al. (1998), who described the formation of electrostatic complexes between lysozyme and other egg white proteins at the air–water interface. However, knowledge about interactions between egg white proteins at the air–water interface is still sparse.

The aim of this work is to gain a better understanding of the conformational changes and the interactions between egg-white proteins that take place at the air–water interface. However, protein structural analyses directly on egg-white are hardly feasible because of the egg-white complexity. In order to reach our objective, we take advantage of our previous work on single-protein systems (Lechevalier et al., 2003), namely, ovalbumin, ovotransferrin and lysozyme, that account for 70% of total egg white proteins (Stadelman & Cotterill, 1977). The study of the three proteins mixed together is expected to give us a closer idea of the mechanisms that are involved at the interface during foaming.

2. Materials and methods

2.1. Reagents

Urea and acrylamide for fluorescence quenching analysis, L-tryptophan and anilino-1-naphthalene-8-sulfonate (ANS), were purchased from Sigma Aldrich. Caesium chloride and potassium iodide were obtained from Merck. 2-Mercaptoethanol and acrylamide for electrophoresis analysis were purchased from Bio-Rad. Sodium dodecyl sulfate (SDS) was purchased from Biosolve Ltd.

2.2. Proteins

Ovalbumin (ova) and ovotransferrin (ovt) were extracted from hen egg white by anion-exchange chromatography according to Croguennec, Nau, Pezennec, and Brulé (2000) and Croguennec, Nau, Pezennec, Piot, and Brulé (2001). Lysozyme (lyso) was obtained from Ovonor (Annezin les Béthune). Proteins were combined in the egg white protein ratio. Total protein concentration was 10 g l^{-1} with 7.7 g l^{-1} ova, 1.8 g l^{-1} ovt and 0.5 g l^{-1} lyso.

2.3. Experimental strategy

Studying the individual structural changes of proteins after foaming in mixtures needed subsequent separation. Since the latter step could also alter protein structure, we studied (in parallel) the effects of separation steps. The experimental strategy is shown in Fig. 1.

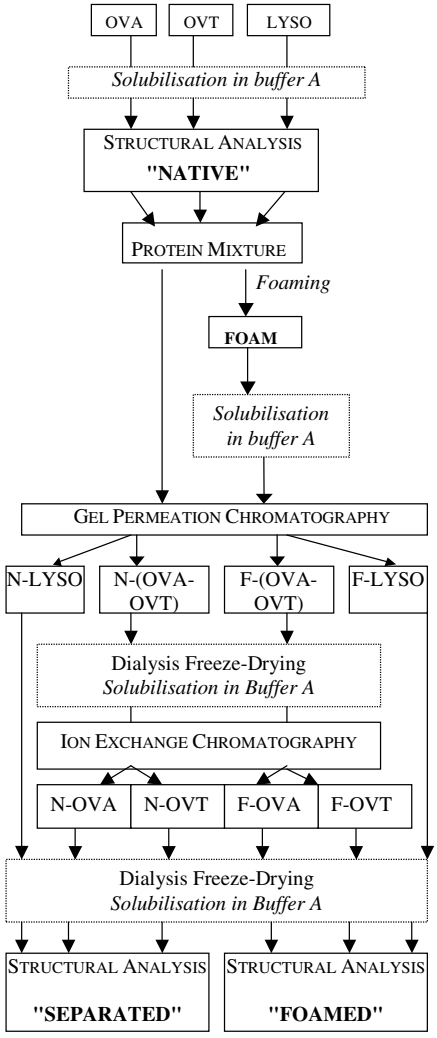

Fig. 1. Experimental strategy used to measure the secondary and tertiary structure changes of ovalbumin, ovotransferrin and lysozyme after foaming in ternary mixture. Proteins deriving from native solution were called N-protein, those deriving from foam were called F-protein.

2.4. Air–water interface generation

Thirty millilitres of a 10 g l^{-1} protein solution dissolved in 67 mM sodium phosphate buffer, pH 7.0 (buffer A), were poured into a bubbling column PM 930 (Grosseron, St Herblain, France), as explained in a previous paper (Lechevalier et al., 2003).

2.5. Solubilisation of proteins from foam

Foam was collected and centrifuged (polypropylene tubes, 3000*g*, 10 min, 20 °C) in order to remove the remaining drained liquid from the foam. Ova–ovt–lyso foam was then solubilized in buffer A.

2.6. Protein separation

To perform structural analyzes, proteins had to be separated. Native solution and solubilized foam solution were first filtered on a 0.2 μm diameter cellulose filter (Minisart RC15, Sartorius, Goettingen, Germany). Lysozyme was first recovered from the protein mixture by gel permeation on a TSK G3000 SWXL (300×7.8 mm^2, i.d.) column (Tosoh Biosep, Stuttgart, Germany) connected to a HPLC system. Proteins were eluted with 0.15 M NaCl in buffer A at a flow rate of 0.8 ml min^{-1}. Since their relatively close molecular weights did not allow separation by gel permeation chromatography, ovotransferrin and ovalbumin were subsequently recovered by anion exchange HPLC on a Q-hyper D10 (100×4.6 mm^2, i.d.) column (Biosepra, Villeneuve-la-Garenne, France) connected to the same HPLC system. Proteins were eluted at a flow rate of 1 ml min^{-1} by a linear gradient of NaCl concentration in buffer A, from 0 to 0.09 M in 12 min. Eluted proteins were collected manually.

Eluted proteins were dialyzed against deionised water and freeze-dried before being resolubilized in buffer A for further separation or analyzes.

2.7. SDS–PAGE analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970), using a 12.5% acrylamide separating gel 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% 2-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. A low molecular weight calibration kit (Amersham Pharmacia Biotech, England) was used. Gels were stained with Coomassie brilliant blue (R250) and destained in ethanol/acetic acid/water (65/25/10 v/v/v) solution. Protein bands were quantified from the scanned gel with the Image Quant software (Amersham Pharmacia Biotech, England).

2.8. Circular dichroism

Circular dichroism (CD)-spectra were obtained using a CD 6 spectropolarimeter (Jobin-Yvon, 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 (Jobin-Yvon, Paris, France). CD-spectra were expressed in terms of molar ellipticity.

$$[\theta_\lambda] = (3300 \times \Delta A_\lambda)/(C \times d), \quad (1)$$

where $[\theta_\lambda]$, is the molar ellipticity at wavelength λ, expressed in deg cm^2 $dmol^{-1}$, ΔA_λ is the difference of absorbance of right- and left-circular polarized light of equal intensity and of the same wavelength λ, C is the mean residue concentration when the far-UV CD-spectrum was reported and the protein molarity in the near UV region, and d (cm) is the light path. α-Helix, β-sheet and random coil structures were determined from the far-UV CD-spectra using the deconvolution software described by Bohm, Muhr, and Jaenicke (1992).

2.9. Fluorescence measurements

Intrinsic fluorescence measurements were performed using a spectrofluorometer LS50B (Perkin–Elmer). Proteins were diluted in buffer A. Protein concentrations were chosen in order to get values in the linearity domain of fluorescence intensity versus protein concentration. Excitation wavelength was 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 at the maximal emission wavelength versus protein concentration by linear regression analysis. The slope was then used as an index of the protein intrinsic fluorescence.

Fluorescence quenching 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), caesium 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 in the presence of quenchers. 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:

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

2.10. Surface hydrophobicity

Measurement of surface hydrophobicity was carried out using the fluorescent probe, ANS. Proteins were diluted in the same way as for the intrinsic fluorescence measurement. Fifteen microlitres of an 8 mM ANS solution were 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 (1980). 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 sample solution was used as a control.

2.11. 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. All analyses were done in triplicate. In all cases, $P \leqslant 0.05$ was considered significant.

3. Results

3.1. Protein composition in foam

SDS–PAGE analysis, under reducing conditions, enabled us to quantify the concentrations of the different proteins at the air–water interface (Fig. 2(A)). Thus, 57% of ovotransferrin, 73% of ovalbumin and 88% of lysozyme present in the native bulk solution (lane 1) were found in foam (lane 2).

3.2. Evidence for covalent aggregation

On SDS–PAGE analysis under non reducing conditions (Fig. 2(B)), bands of dimeric ovalbumin, as well as of higher molecular weight aggregates, were visible for the native solution (lane 1). 28% of ovalbumin was then in polymeric form, including 15% in dimeric form. In the foam fraction, the intensity of these bands, and also of those of monomeric proteins, decreased and

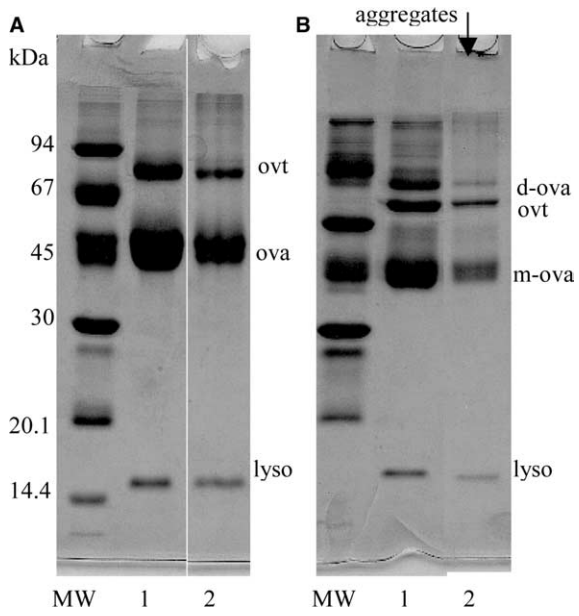

Fig. 2. SDS–PAGE with (A) and without (B) 2-mercaptoethanol of ova–ovt–lyso native solution (lane 1) and ova–ovt–lyso foam fraction (lane 2); molecular weight (MW).

bands of aggregates, remaining at the top of stacking gel, appeared (lane 2). Upon treatment with 2-mercaptoethanol, these aggregates were dissociated in monomeric proteins (Fig. 2(A), lane 2). Around 64% of lysozyme, 65% of ovalbumin and 60% of ovotransferrin, present in foam, were then involved in high molecular weight aggregates formation.

3.3. Strategy to study the structure of remaining proteins in monomeric form

In foam, the initial protein concentration ratio was not kept constant. Ova–lyso and ovt–lyso ratios decreased, indeed, from 15.4 to 12.8 and from 3.6 to 2.3, respectively, which assumed lysozyme concentration was constant in foam. On the other hand, the ova–ovt ratio increased from 4.3 to 5.5, assuming ovotransferrin partial exclusion from the air–water interface. This result did not allow us to apply structural analyzes directly on the protein mixtures. Actually, effects due to protein structure modifications could be confused with those due to changes in protein composition and concentration. This is the reason why we chose to separate the proteins before further analyzes, though separative procedures used could add structural modifications. However, thanks to the protocol we adopted (Fig. 1), it was possible to distinguish the effects due to separation from those due to foam formation.

3.4. Separation protocol may damage protein structure

Lysozyme was separated from ova–ovt by gel permeation chromatography. It was then lyophilised and solubilised in buffer A. Its structure was modified by this

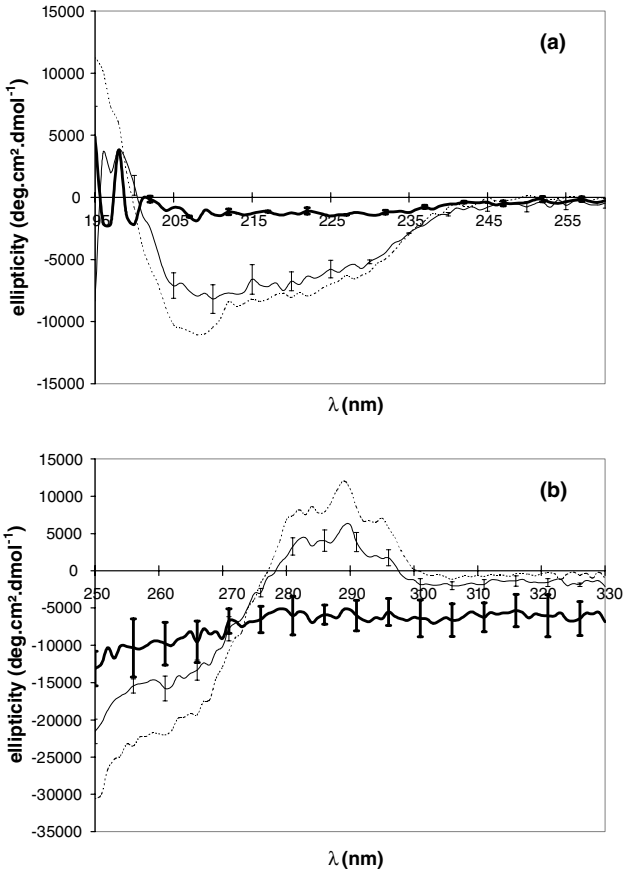

Fig. 3. Far- (a) and near- (b) UV CD-spectra of lysozyme: Dotted line shows the spectra for native lysozyme, solid thin line shows the spectra for lysozyme recovered from native solution, as described in Fig. 1 and solid thick line shows the spectra for lysozyme recovered from foam fraction, as described in Fig. 1.

protocol. Its far-UV CD spectrum was, indeed, different after separation (Fig. 3(a)). These changes corresponded to a loss of 47% (±14) of α-helix structures in favour of random coil structures (+45%, ±16). These secondary structure modifications led to tertiary structure changes, as suggested by the differences observed between near-UV CD spectra before and after separation (Fig. 3(b)). However, remaining peaks in the tyrosine and tryptophyl residue adsorption range suggested that tertiary structure was partly preserved. The results of intrinsic and quenching fluorescence reinforced this hypothesis. Only a slight blue shift (−1 nm, ±0.8) was, indeed, observed after excitation at 295 nm, whereas no shift occurred after excitation at 280 nm (Table 1). The accessibility of tryptophyl residues to the different quenchers was also modified (Table 2). Nevertheless, surface hydrophobicity increased fivefold (Table 3).

Ovotransferrin underwent gel permeation and ion-exchange chromatographies, as well as two steps of freeze-drying. This protocol did not damage its secondary structure since the far-UV CD-spectrum was not significantly different from the native protein one (Fig. 4(a)). Only a slight increase in the proportion of β-turn structures occurred (+6%, ±4). On the other hand, tertiary structure was modified, as suggested by the near-UV CD-spectrum (Fig. 4(b)). The environment of sulfhydryl residues (between 250 and 260 nm) was the most modified whereas remaining peaks in the adsorption wavelength range of tyrosine and tryptophyl residues were still visible. However, the environment of the latter two residues was even so changed, since significant red shifts were noticed in the intrinsic fluorescence (Table 1), as well as a twofold higher number of tryptophyl residues accessible to iodide (Table 2). At the same time, surface hydrophobicity increased (Table 3).

Ovalbumin underwent the same separation protocol as ovotransferrin. Nevertheless, the effects on its structure were stronger. Ovalbumin secondary structure was, indeed, strongly damaged (Fig. 5(a)). The protein

Table 1
Shifts (nm) in the maximal emission wavelength observed in intrinsic fluorescence after excitation at 280 and 295 nm for lysozyme, ovotransferrin and ovalbumin recovered from native solution (“separated”) and from foam fraction (“foamed”), as described in Fig. 1

λex nm	Lysozyme		Ovotransferrin		Ovalbumin	
	Separated	Foamed	Separated	Foamed	Separated	Foamed
280	NS	NS	+1.9 (±1.0)	NS	NS	NS
295	−1 (±0.8)	NS	+2.2 (±1.3)	NS	−1.4 (±1.0)	NS

NS, non significant.

Table 2
Variation (%) in the accessibility of tryptophyl residues to the three quenchers for lysozyme, ovotransferrin and ovalbumin recovered from native solution (“separated”) and from foam fraction (“foamed”), as described in Fig. 1

	Lysozyme		Ovotransferrin		Ovalbumin	
	Separated	Foamed	Separated	Foamed	Separated	Foamed
Acrylamide	−52 (±15)	NS	NS	+57 (±17)	+100 (±33)	−37 (±23)
Caesium	+14 (±10)	NS	NS	+100 (±51)	+88 (±80)	NS
Iodide	−61 (±8)	NS	+111 (±68)	NS	+152 (±84)	NS

NS, non significant.

Table 3
Variation (%) in the surface hydrophobicity for lysozyme, ovotransferrin and ovalbumin recovered from native solution (“separated”) and from foam fraction (“foamed”), as described in Fig. 1

	Lysozyme		Ovotransferrin		Ovalbumin	
	Separated	Foamed	Separated	Foamed	Separated	Foamed
Surface hydrophobicity	+410 (±67)	+65 (±39)	+26 (±8)	−47 (±13)	−91 (±4)	−81 (±38)

NS, non significant.

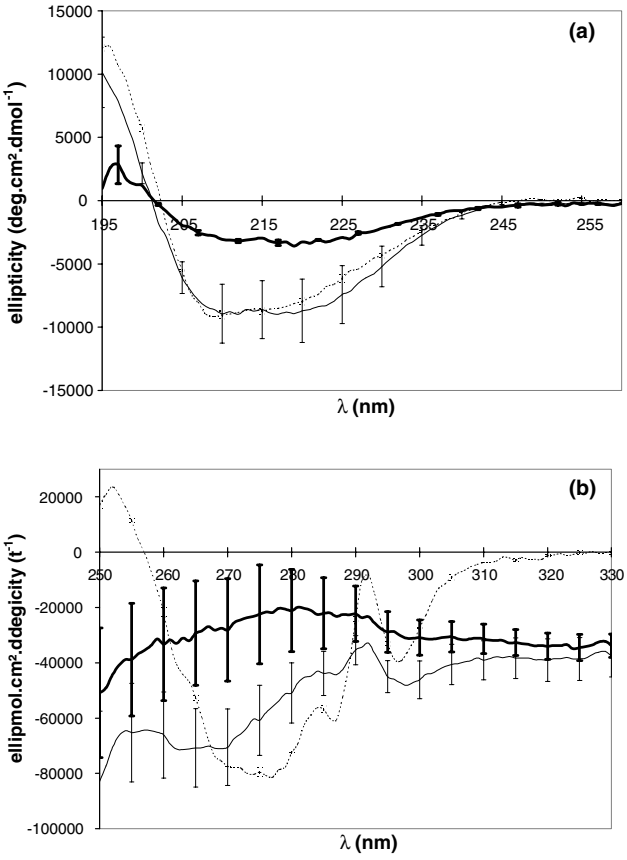

Fig. 4. Far- (a) and near- (b) UV CD-spectra of ovotransferrin: Dotted line shows the spectra for native ovotransferrin, solid thin line shows the spectra for ovotransferrin recovered from native solution, as described in Fig. 1 and solid thick line shows the spectra for ovotransferrin recovered from foam fraction, as described in Fig. 1.

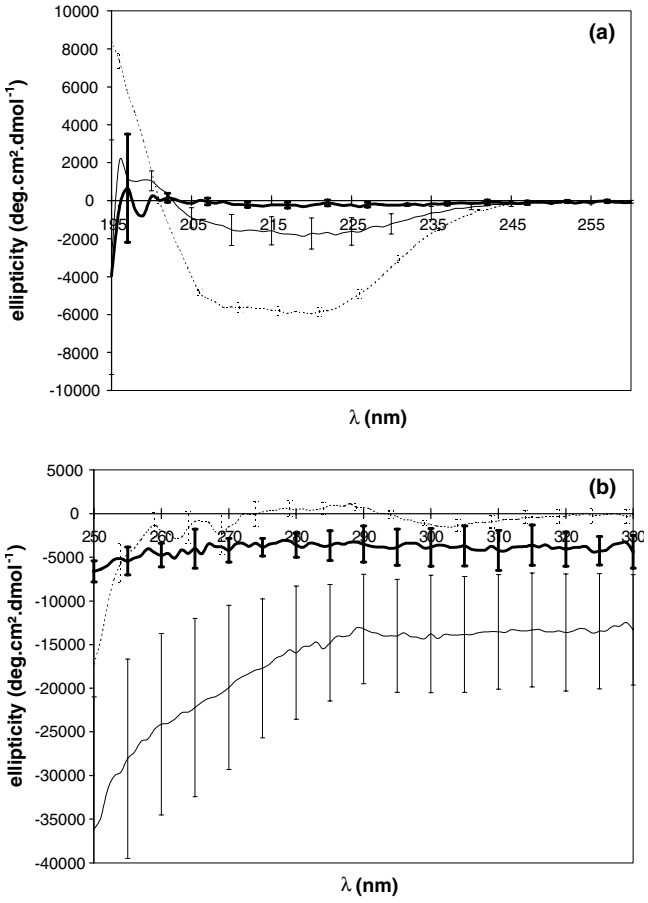

Fig. 5. Far- (a) and near- (b) UV CD-spectra of ovalbumin: Dotted line shows the spectra for native ovalbumin; solid thin line shows the spectra for ovalbumin recovered from native solution, as described in Fig. 1 and solid thick line shows the spectra for ovalbumin recovered from foam fraction, as described in Fig. 1.

lost 67% (±11) of α-helix structures in favour of β-sheet and random coil structures (+48 (±14) and +12% (±4), respectively). Tertiary structure was then also strongly damaged (Fig. 5(b)). And yet, only a slight blue shift in intrinsic fluorescence after excitation at 295 nm was noticed (Table 1) but the accessibility of tryptophyl residues to the three quenchers was strongly modified (Table 2). Moreover, surface hydrophobicity decreased dramatically (Table 3).

3.5. Effects of the air–water interface on proteins structure

In this section, the results obtained for “foamed” proteins were compared to those obtained for “separated” proteins.

Lysozyme secondary structure was strongly modified after contact with the air–water interface (Fig. 3(a)). The protein lost 55% (±37) of the remaining α-helix structures as compared to “separated” lysozyme. Secondary and tertiary structures were completely lost (Fig. 3(b)). However, no further significant shift in intrinsic fluorescence or changes in tryptophyl residue accessibility to quenchers were noticed (Tables 1 and 2, respectively). Only, a significant increase of surface hydrophobicity was determined (Table 3).

Ovotransferrin secondary structure did not undergo any change after separation but was strongly modified after contact with the air–water interface (Fig. 4(a)).

The protein lost 69% (±32) of α-helix structures in favour of β-sheet and random coils structures (+61 (±30) and +6% (±2), respectively). Tertiary structure was then completely lost (Fig. 4(b)). Though no significant shift was observed in intrinsic fluorescence (Table 1), accessibility of tryptophyl residues to acrylamide and caesium increased (Table 2), whereas surface hydrophobicity decreased (Table 3).

Ovalbumin was denatured after separation. After contact with the air–water interface, the protein was further damaged, as suggested by the far-UV CD-spectrum (Fig. 5A). A further loss of 54% (±15) of α-helix structures and 84% (±4) of β-sheet structures was determined in favour of β-turn and random coil structures (+30 (±5) and +80% (±4), respectively). Tertiary structure was completely lost (Fig. 5(b)). No further shift was observed in intrinsic fluorescence (Table 1) but tryptophyl residues were less accessible to acrylamide (Table 2) and surface hydrophobicity decreased (Table 3).

4. Discussion

The exposure of ovalbumin, ovotransferrin and lysozyme in mixture to air–water interface leads to the formation of high molecular weight covalent aggregates. Around 60% of each protein is thus covalently bound. Poole, West, and Clifford (1984) suggested that lysozyme was involved in foam formation of egg albumen through electrostatic interactions with negatively charged proteins, such as ovalbumin at pH 7. Damodaran et al. (1998) demonstrated the existence of an electrostatic complex between lysozyme and other egg white proteins, even at high ionic strength, if protein concentrations were high enough. They also showed that ovalbumin was the first protein to diffuse to the air–water interface, ovotransferrin and lysozyme showing a lag phase. We showed in a previous work that, once exposed to the air–water interface in a single-protein system, ovalbumin unfolded irreversibly and formed large aggregates through disulfide bond formation (Lechevalier et al., 2003). Thus, in mixture, ovalbumin unfolded at the interface, could expose its free sulfhydryl groups in a similar way, and then react with ovalbumin and other proteins at the interface, namely ovotransferrin and lysozyme, through intermolecular sulfhydryl–disulfide exchanges, thus explaining the aggregation observed.

Such sulfhydryl–disulfide exchanges were already noticed by Xu, Shimoyamada, and Watanabe (1998), Watanabe, Nakamura, Xu, and Shimoyamada (2000) and Matsudomi, Oka, and Sonoda (2002), between ovalbumin denatured by dry-heating or pre-heating treatments and ovotransferrin. Matsudomi et al. (2002) also suggested that heat-induced aggregation between lysozyme and ovalbumin resulted from electrostatic interactions and disulfide bond interchanges, with electrostatic interactions being the prominent factor if denatured ovalbumin was used (Matsudomi, Yamamura, & Kobayashi, 1986).

It is also notable that the proximity, due to electrostatic interactions, of the three proteins favours the formation of these covalent aggregates compared to ovalbumin polymers alone, since the corresponding bands intensity in SDS–PAGE analysis decreased.

According to SDS–PAGE analysis results, around 40% of the proteins are still in the monomeric form in the foam. In order to avoid confusion between structural modifications and concentration ratio changes, and also in order to compare the results with those obtained in a previous work, with single-protein systems (Lechevalier et al., 2003), the study of monomeric protein structure requires their separation.

To do so, we chose chromatographic techniques, often used to purify proteins from egg albumen, that are not supposed to damage protein structure. Nevertheless, some chromatographic techniques seem to be less denaturing than others. For example, Awade, Moreau, Molle, Brulé, and Maubois (1994) mentioned that lysozyme purified using gel permeation chromatography had a better enzymatic activity than the one purified using ion-exchange chromatography.

In our case, lysozyme structure is modified. However, the first step of separation, i.e., gel permeation chromatography, dilutes eluted proteins and lyophilization is then required before further analyzes. Constantino, Griebenau, Mishra, Langer, and Klibonov (1995) showed that freeze-drying damaged lysozyme secondary structure with a loss of α-helix structures, which was similar to our measurements. Poole and Finney (1983) also demonstrated that the rehydration step was a critical point with a possible "loosening up" of the protein.

Ovotransferrin secondary structure is not damaged after separation. Only tertiary structure is partly lost. Red shifts in intrinsic fluorescence, exposure of tryptophyl residues to fluorescence quenchers and increase in surface hydrophobicity revealed the unfolding of the protein. Possibly its well-known tertiary structure flexibility protects it from any further denaturation.

Conversely, ovalbumin is much more damaged after the same separation steps as ovotransferrin. Its secondary structure moves toward β-sheet structures. Tryptophyl residues are exposed, as suggested by their increasing accessibility to fluorescence quenchers, whereas hydrophobic residues are masked. Once again, freeze-drying seems responsible for these structural changes. Kitabatake, Indo, and Doi (1989) showed, indeed, that freeze-drying improved ovalbumin foaming properties and slightly modified its far-UV CD spectrum. Constantino et al. (1995) found similar results after lyophilization of human albumin.

Separation techniques used thus have significant effects on protein structure. Ovotransferrin undergoes limited tertiary structure changes. Both tertiary and secondary structures of lysozyme are damaged but without complete unfolding. Ovalbumin is the more sensitive with dramatic loss of secondary and tertiary structures.

Structural modifications undergone by ''foamed'' proteins are comparable to those obtained for ''separated'' proteins. Differences were assigned to protein contact with the air–water interface.

Lysozyme structure changes are stronger after foaming and separation than after separation only. UV CD-spectra showed that both tertiary and secondary structures were lost. Moreover, secondary structure changes are not of the same nature: after separation, the loss of α-helix structures was in favour of random coil structures whereas, after foaming, it was in favour of β-sheet structures. Concerning tertiary structure, conformational changes between ''separated'' and ''foamed'' lysozyme were not very different. The only difference was the surface hydrophobicity increase. Obviously, lysozyme structure changed after foaming. In a previous work (Lechevalier et al., 2003), we showed that in a single-protein system, lysozyme structure was not damaged at the air–water interface. If this were the case in mixture, such differences between secondary structures of ''foamed'' and ''separated'' lysozyme would not have been obtained. After foaming in the presence of ovalbumin and ovotransferrin, lysozyme, in monomeric form, is at least partly denatured.

Ovotransferrin structure was slightly damaged after separation. On the other hand, after foaming and separation, secondary and tertiary structures were strongly modified. No further shifts were noticed in intrinsic fluorescence, but tryptophyl residues were more accessible to acrylamide and caesium. Surface hydrophobicity increased after separation, but decreased after foaming and separation. It is thus very likely that ovotransferrin structure had changed after foaming. This is not very surprising, since we showed in previous work (Lechevalier et al., 2003) that, after contact with the air–water interface in single-protein system, ovotransferrin unfolded. This is also the case with foaming in mixture, since results were very similar.

Ovalbumin structure was strongly damaged by the separation protocol. Nevertheless, after foaming, it was again further altered. Especially, secondary structure modifications were different. After separation only, the loss of α-helix structures was in favour of β-sheet and random coil structures whereas, after foaming and separation, there was a loss of α-helix and β-sheet structures in favour of β-turn and random coil structures. On the other hand, tertiary structure changes were not very different, except for a greater loss of surface hydrophobicity after foaming. Contact with the air–water interface in the presence of ovotransferrin and lysozyme is thus not without consequences on ovalbumin structure. In the single-protein system, ovalbumin unfolded at the air–water interface before aggregating (Lechevalier et al., 2003). In mixture, structural modifications were different, since the protein then had an unordered secondary structure instead of a β-sheet structure.

This study was aimed at contributing to a better understanding of egg white protein behaviour in mixture at the air–water interface. However, available protein structure analyses require single-protein systems, implying separation steps from protein mixtures. According to the literature (Awade et al., 1994), the present results show that protein separation using chromatography damaged protein structure, but no alternative preparative method exists for protein separation. In the same way, protein concentration, using lyophilisation, modified protein structure. Other techniques for concentration, such as ultrafiltration or vacuum-evaporation could be considered for further study, but without any guarantee to be less denaturing.

Despite the impact of the protein preparation methods, the foamed protein structure was definitely different from the separated protein one. This demonstrated an effect of the air–water interface on protein structure. However, the nature and intensity of denaturation, due to the interface, were not determinable since the effects of separation could differ according to the initial protein conformation (native or modified by the air–water interface).

Simultaneous presence of ovalbumin, ovotransferrin and lysozyme in the bulk during foaming is responsible for the establishment of a ''synergy of denaturation''. Lysozyme that was not damaged in single-protein systems (Lechevalier et al., 2003), was completely unfolded in the monomeric soluble form or involved in covalent aggregates. In the single-protein system, ovotransferrin was admittedly completely unfolded but it stayed in the monomeric form (Lechevalier et al., 2003); in mixture, most of it was involved in covalent aggregates. Ovalbumin already aggregates in the single-protein system but in smaller proportions (Lechevalier et al., 2003). Interactions and synergies that occur in a multi-proteic system prevent any extrapolation from the results obtained with model solutions.

Acknowledgements

The authors thank Dr. Bouhallab for helpful discussions. The authors are grateful to M. Piot for technical assistance. This work was supported by the Conseil Régional de Bretagne and the Conseil Régional des Pays de la Loire.

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