Modifications of Interfacial Proteins in Oil-in-Water Emulsions Prior to and During Lipid Oxidation

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Ⓢ Supporting Information

ABSTRACT: Lipid oxidation is a major cause for the degradation of biological systems and foods, but the intricate relationship between lipid oxidation and protein modifications in these complex multiphase systems remains unclear. The objective of this work was to have a spatial and temporal insight of the modifications undergone by the interfacial or the unadsorbed proteins in oil-in-water emulsions during lipid oxidation. Tryptophan fluorescence and oxygen uptake were monitored simultaneously during incubation in different conditions of protein-stabilized oil-in-water emulsions. Kinetic parameters demonstrated that protein modifications, highlighted by decrease of protein fluorescence, occurred as an early event in the sequence of the reactions. They concerned more specifically the proteins adsorbed at the oil/water interface. The reactions led in a latter stage to protein aggregation, carbonylation, and loss of protein solubility.

KEYWORDS: lipid oxidation, emulsion, interface, protein, front-surface fluorescence, protein carbonylation, aggregation

INTRODUCTION

Oxidation of polyunsaturated fatty acids (PUFA) is a chemical reaction which decreases the nutritional and sensory properties of food products.[1] It also contributes to the oxidative stress in vivo.[2-5] In these complex and multicomponent systems, lipid oxidation generally occurs simultaneously with "co-oxidation" phenomena, which affect other molecules such as proteins.[6-8] Protein oxidation is involved in various human diseases and aging[9,10] but also in the degradation of the sensory properties of food products such as texture[8,11,12] and in the loss of protein digestibility.[12-14]

Since the 1970s, it is known that the proteins present in foods and food emulsions are susceptible to be attacked by the free radicals, hydroperoxides, and secondary lipid oxidation products as aldehydes, leading to the formation of various reaction products.[6,7] For the last 10 years, the question of the oxidative modifications of proteins in biological systems, food products, and related food models has been identified as an emerging subject. It addresses the nutritional, toxicological, and possibly sensory consequences of protein modifications in vivo and in the food products.[8] Indeed, the intricate radical and non radical mechanisms involving both lipid oxidation and protein modifications have not been fully elucidated yet.

Time dependence between lipid and protein oxidations was observed in multiphase systems. The two phenomena were described as "correlated",[15] "concomitant",[16] or "simultaneous".[17] Lund et al. also reported a timely coincidence between lipid and protein oxidation in muscle foods.[12] It seems therefore obvious that lipid and protein oxidation are linked, but it is difficult to figure out which of the phenomena first starts. On the one hand, proteins present in food emulsions are susceptible to be attacked by the free radicals, hydroperoxides, and secondary products as aldehydes resulting from lipid oxidation, leading to the formation of various reaction products.[6,7,15,18,19] On the other hand, fatty acid oxidation can be induced by bovine serum albumin (BSA) radicals[20] and

amino acid residues of β-lactoglobulin (BLG) were oxidized prior to the propagation of lipid oxidation in O/W emulsion.[21]

The location of proteins and their respective concentrations in one or in the other phase have also to be taken in account when oxidation in multiphase systems is considered. In O/W emulsions, proteins are either adsorbed at the interface surrounding the oil droplets or, once the emulsifiers have covered the interface, unadsorbed in the aqueous phase. Rampon et al. revealed by front-surface fluorescence measurements that during oxidation of BSA-stabilized O/W emulsions unadsorbed BSA was by far less modified than BSA adsorbed onto the oil droplets.[22] It was also repeatedly found that when the concentration of unadsorbed proteins increased, lipid oxidation slowed down.[23-26] However, these studies explain only partially the links between the protein location within multiphase systems, lipid oxidation, and protein modifications.

To have a better insight on the relationship between protein and lipid oxidation in protein-stabilized emulsions, we have determined the extent and kinetics of modifications of interfacial and unadsorbed proteins in oxidizing oil-in-water emulsions in relation to lipid oxidation. Lipid oxidation and protein modifications undergone by the interfacial and the unadsorbed proteins were assessed in O/W emulsions stabilized by either BLG, β-casein (BCN), or BSA with limited amounts of proteins remaining in the aqueous phases. The oxidizing conditions were the same as applied in our previous work.[23,27]

MATERIALS AND METHODS

Materials. Rapeseed oil was purchased in a local supermarket. It was stripped by means of alumina (MP Alumina N-Super I, MP

Table 1. Physical Characteristics of O/W Emulsions Stabilized with Different Proteins[a]

		protein concentration (g L^{-1})			
emulsion code	excess protein	in aqueous solution (initial)	in aqueous phase of emulsion at t_0	in aqueous phase of emulsion at $t = 48$ h	$[d_{3,2}]$ at t_0 (μm)
BLG		5.00	1.52 ± 0.09	1.16 ± 0.14	1.5 ± 0.1
BLG+	BLG	10.00	6.05 ± 0.10	5.77 ± 0.15	1.5 ± 0.1
BSA		4.00	1.17 ± 0.11	0.98 ± 0.03	1.8 ± 0.1
BCN		5.00	0.45 ± 0.12	0.99 ± 0.03	1.7 ± 0.1
BCN+	BCN	10.00	3.60 ± 0.10	3.94 ± 0.17	1.9 ± 0.1

[a]Emulsions contained 30 g of stripped rapeseed oil and 70 g of protein solution in pH 6.7 buffer (PIPES 10 mM, NaCl 80 mM). Mean and standard deviation of three measurements on three emulsions were prepared independently. Data at $t = 48$ h have been determined after 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. Data taken from Berton et al.[23,28] Abbreviations: BLG = β-lactoglobulin; BCN = β-casein; BSA = bovine serum albumin; EDTA = ethylene diamine tetraacetic acid; $[d_{3,2}]$: volume−surface diameter.

Biomedicals, France) to eliminate impurities and tocopherols.[28] Stripped oil contained less than 2 μg of residual tocopherols per g of oil and 0.34 μmol of hydroperoxides per g of oil. Its triacylglycerols contained as major fatty acids: oleic acid (C18:1 n-9; around 62% of total identified fatty acids), linoleic acid (C18:2 n-6; 19.7%), and linolenic acid (C18:3 n-3; 9.2%) and less than 7% saturated fatty acids (mainly palmitic acid). BSA (fraction V, minimum 96% by agarose gel electrophoresis) was obtained from MP Biomedicals (France). BCN (purity ≥98%) was purchased from Lactalis (France). β-Lactoglobulin (BLG) has been purified at the laboratory from Prolacta 90 (Lactalis) by selective precipitation as previously described.[28] 1,4-Piperazinediethanesulfonic acid (PIPES), ethylene diamine tetraacetic acid disodium calcium salt (EDTA), and iron(II) sulfate (FeSO$_4$) were purchased from Sigma Aldrich (France). NaCl and SDS were purchased from Fluka Chemika (France). All other chemicals and solvents were at least of analytical grade and purchased from Sigma Aldrich (France) or Carlo Erba (France). The buffer was composed of PIPES (10 mM) and NaCl (80 mM) and adjusted at pH 6.7.

Preparation and Characteristics of Emulsions. The O/W emulsions were prepared according to the already described procedure.[23,27,28] A first set of emulsions was characterized by concentrations of unadsorbed proteins as low as possible. A second set that contained an excess of protein in the aqueous phase was prepared in two steps according to the procedure described in Berton et al. 2011.[23] The concentration of unadsorbed proteins in the aqueous phase at t_0 and after 48 h of incubation was measured in the two sets as previously described.[28] Table 1 reminds the main characteristics of these systems as previously detailed.[23] The volume−surface mean diameter ($[d_{3,2}]$) of the emulsions was comprised between 1.4 and 2.2 μm.[28] The initial level of oxidation of the freshly prepared emulsions was evaluated by the measurement of conjugated dienes. CD were undetected in the BSA- and BCN-stabilized emulsions and remained at very low levels in BLG-stabilized emulsions (<5 μmol equiv HPX g^{-1} oil).

Incubation of Emulsions. The emulsions were added either one volume of one of the following oxidation initiators (final concentration in the emulsion): FeSO$_4$/EDTA (1/1, M/M, 200 μM) prepared as described in Berton et al.,[23] metmyoglobin (1 μM), FeCl$_3$/sodium ascorbate (1/1, M/M, 50 μM), 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH; 1 mM),[27] or the same volume of ultrapure water. Aliquots (3 mL) of the emulsions were distributed in 20.5 mL headspace vials sealed with Teflon/silicon septa and aluminum crimp caps. The vials were then rotated at 5 rpm in the dark, either at 25 °C when the one or the other oxidation initiator was used or at 33 °C when only ultrapure water was added.

Oxygen Uptake in Oxidizing O/W Emulsions. Oxygen uptake measured in hermetically sealed vials is a relevant marker of lipid oxidation.[23,29,30] We used the estimated kinetic parameters obtained by the adjustment of the experimental data obtained in Berton et al.[23,27] The kinetic parameters include the lag period before the beginning of oxygen uptake (L_{O2}, h), the oxygen uptake rate (μ_{O2}, mmol O$_2$ kg^{-1} oil h^{-1}), and the incubation time when half the maximum oxygen uptake was reached ($t_{1/2,\ O2}$, h). Additionally, oxygen was measured in the headspace of protein solutions (BLG, BCN or

BSA, 6 g L^{-1} pH 6.7 PIPES buffer, FeSO$_4$/EDTA 200 μM) during their incubation at 25 °C in the same conditions as the emulsions.

Recovery of Aqueous and Creamed Phases of Emulsions. The aqueous and the creamed phases of emulsions were recovered as previously described.[28] Briefly, aliquots of the emulsions were centrifuged (3500g, 45 min, 20 °C) to separate the aqueous phases from the creamed phases containing the oil droplets covered by the adsorbed protein. The aqueous phases were collected and sequentially filtered through 0.45, 0.20, and 0.10 μm cellulose acetate filters (Minisart High-Flow, Sartorius, Germany) to remove the residual small oil droplets. The creamed phase was collected separately. The amount of proteins in each phase was determined according to the method described by Markwell et al.[31]

Front-Surface Fluorescence Spectroscopy. The evolution of the fluorescence signal of proteins during incubation of emulsions was followed in situ in front-surface fluorescence mode.[22,32] Front-surface fluorescence spectra were obtained with a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) fitted with a variable angle front-surface accessory set at 56°. Excitation and emission slit widths were set at 5.0 and 2.5 nm, respectively. Approximately 140 μL of sample (whole emulsion, aqueous phase, or creamed phase) were put in a quartz cell with a 0.5 mm optical pathway (Hellma, 106 QS). Tryptophan (Trp) emission spectra were acquired between 290 and 350 nm, with an excitation wavelength set to 290 nm, a scan speed of 240 nm min^{-1}, and steps of 0.2 nm. Experiments were performed in a controlled-temperature room (22 ± 3 °C). Results were expressed in signal intensity at 334 nm (I) normalized with the intensity at t_0 (I_0).

Extraction of Proteins. Two hundred microliters of sample (whole emulsion, aqueous phase, or creamed phase) were introduced in 2 mL Eppendorf tubes. The proteins were precipitated and washed twice by addition of 1800 μL of isopropyl alcohol. The tubes were shaken and centrifuged (14000g, 5 min, 10 °C). The supernatants were discarded, and the washed proteins were stored at −20 °C until further analysis.

Protein Solubility and Protein-Bound Carbonyls. Protein carbonyls were determined after their solubilization in guanidine chloride (GuCl) 6 M. Therefore, the solubility of proteins from whole emulsions, aqueous phase, or creamed phase of emulsions was determined as follows: proteins recovered as described above were dispersed in 500 μL 2N HCl solution for 60 min in the dark. Proteins were precipitated again with 500 μL of 400 g L^{-1} trichloroacetic acid solution for 10 min in ice. The tubes were centrifuged (14000g, 5 min, 10 °C), and the supernatants were discarded. The pellets were washed twice with 1 mL of ethanol/ethyl acetate 1/1 v/v, once with 1 mL of isopropyl alcohol, and dissolved in 1 mL of guanidine chloride 6 M at 37 °C for 30 min to 12 h. The tubes were finally centrifuged (14000g, 3 min, 10 °C) to eliminate the insoluble fraction, if any. The amount of GuCl 6 M-soluble proteins was determined in the supernatants using the bicinchoninic acid assay microtitration (BC Assay)[33] coupled with calibration curves established with standard solutions for each protein (BLG, BCN, or BSA). Results were expressed as percentage of soluble proteins as compared to the t_0 value.

Protein carbonyls were determined according to the procedure described by Levine et al.[34] The method is based on the derivatization
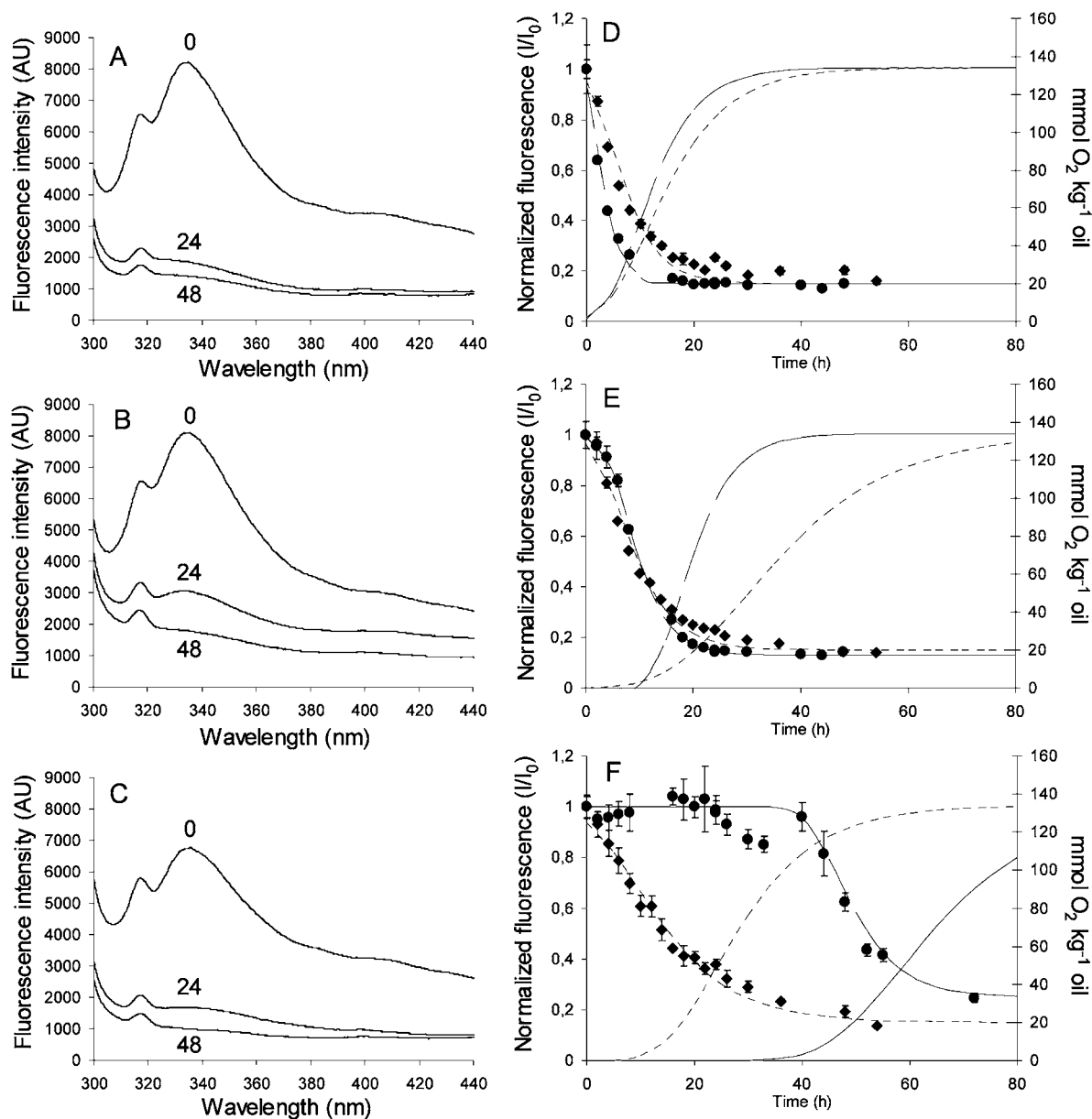
Figure 1. Evolution of protein fluorescence and oxygen uptake in the protein-stabilized oil-in-water (O/W) emulsions at pH 6.7 without excess of protein. Left: Fluorescence spectra ($\lambda_{\text{excitation}}$ = 290 nm) of (A) BLG, (B) BCN, or (C) BSA in emulsions freshly prepared (t = 0) or after 24 or 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. Right: Normalized Trp fluorescence ($\lambda_{\text{excitation}}$ = 290 nm, $\lambda_{\text{emission}}$ = 334 nm) (curves with symbols) and oxygen uptake (curves without symbols, data from Berton et al.[23]) in emulsions stabilized with (D) BLG, (E) BCN, or (F) BSA incubated at 33 °C (●, ——) or at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM (◆, ---). Curves correspond to the modified Gompertz equation with the estimated L and μ parameters for each emulsion. Errors bars represent standards deviations (n = 3 to 6 for fluorescence data; n = 6 to 12 for oxygen uptake data). Abbreviations: BLG = β-lactoglobulin; BCN = β-casein; BSA = bovine serum albumin; EDTA = ethylene diamine tetraacetic acid; Trp = tryptophan; L = lag phase (h); μ = reaction rate (h^{-1}).

of carbonyls with 2,4-dinitrophenylhydrazine (DNPH). Three test tubes were prepared for each sample. The protocol was this described above for the determination of protein solubility, except that total proteins were first dispersed in 500 μL of 10 mM DNPH solution in 2N HCl. The absorbance of the supernatants was measured at 370 nm, the control supernatants (without DNPH) being used as blanks. The protein-bound carbonyl content was calculated using a molar absorption coefficient of 22000 M^{-1} cm^{-1}.[34] Results were expressed in μmol carbonyl per g of soluble proteins.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under either non-reducing or reducing conditions. Protein pellets obtained from isopropyl alcohol precipitation were dissolved in a pH 6.8-buffer constituted of Tris 0.5 M, SDS 10%, glycerol 30%, and bromophenol

blue. The volume of buffer added in each sample (whole emulsion, aqueous phase, or creamed phase) was determined to obtain a theoretical concentration of 2 mg mL^{-1} protein in the t_0 samples, as expected from the initial amounts of proteins used to prepare the emulsion samples. β-Mercaptoethanol (8%) was added when reducing conditions were applied. Samples were vortexed, incubated at room temperature for 1 h 30 min, and heated at 95 °C for 5 min. Then, either 10 μL of a molecular weight marker (Bio-Rad, range 161−0305) or 10 μL of protein solution were brought onto a 10%- (BSA) or 12%- (BLG, BCN) polyacrylamide gel coupled with a pH 8.3 migration buffer constituted of Tris 25 mM, glycine 192 mM, and SDS 0.1%. Electrophoresis was performed at 20 mA, and the gels were stained with a Coomassie blue solution. Gels were finally scanned. The intensity of the monomer band was then measured with the

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Table 2. Lag Periods (L_{Trp} and L_{O2}) and Rates of the Decrease of Normalized Trp Fluorescence (μ_{Trp}) and of Oxygen Uptake (μ_{O2}) for the Emulsions Incubated in Different Conditions[a]

emulsion[c]	incubation condition		parameters of Trp fluorescence				parameters of oxygen uptake[b]			
	catalyst	T (°C)	L_{Trp} (h)	μ_{Trp} (h^{-1}), × 10^2	R^2	$t_{1/2, Trp}$ (h)	L_{O2} (h)	μ_{O2} (mmol O$_2$ kg^{-1} oil h^{-1})	R^2	$t_{1/2, O2}$ (h)
BLG	Fe/EDTA	25	0.2 ± 0.9	6.8 ± 1.0	96.0	6.5	3.3 ± 0.4	6.1 ± 0.2	98.3	14.4
BLG+	Fe/EDTA	25	~0	3.7 ± 0.4	96.4	8.8	9.9 ± 1.3	3.9 ± 0.3	95.7	27.3
BLG	none	33	~0	14.4 ± 1.5	98.6	3.0	2.3 ± 0.3	8.0 ± 0.3	99.3	12.1
BCN	Fe/EDTA	25	1.0 ± 0.8	5.8 ± 0.7	97.8	8.4	15.0 ± 0.8	3.3 ± 0.1	96.9	35.6
BCN+	Fe/EDTA	25	~0	1.3 ± 0.2	83.2	25.1	44.4 ± 1.2	3.9 ± 0.4	94.4	61.8
BCN	none	33	3.2 ± 0.3	7.3 ± 0.4	99.7	9.2	12.4 ± 0.5	9.2 ± 0.5	99.2	20.9
BSA	Fe/EDTA	25	~0	3.2 ± 0.4	96.9	12.9	14.0 ± 0.8	4.8 ± 0.3	97.1	27.9
BSA	none	33	40.3 ± 2.0	4.9 ± 1.3	91.1	47.9	44.4 ± 1.5	3.4 ± 0.3	91.1	67.2

[a]Asymptote confidence intervals for L and μ values were calculated with a 95% significance level. $t_{1/2, Trp}$ and $t_{1/2, O2}$ values are incubation times calculated with the model when half the minimum Trp fluorescence or maximum oxygen uptake were reached, respectively. Correlation coefficients (R^2) indicate the percentage of dispersion explained by the adjusted model. Abbreviations: BLG = β-lactoglobulin; BCN = β-casein; BSA = bovine serum albumin; EDTA = ethylene diamine tetraacetic acid. [b]Data from Berton et al.[23,27] [c]Emulsion code: see Table 1.

MultiGauge software with the background noise subtracted for each band and expressed as a percentage of the intensity of the band at t_0. For each protein, electrophoresis characterization was performed in triplicate.

Molar Balance of Substrates and Reaction Products in Oxidized Emulsions. The molar balance between oxidation substrates (oxygen, PUFA) and oxidation products (conjugated dienes: CD), volatile compounds, and protein carbonyls) was established in the BLG-stabilized emulsion after 30 h of incubation at 25 °C with FeSO$_4$/EDTA (1/1, M/M, 200 μM). The molar amount of PUFA in rapeseed oil and lipid phase extracted from the oxidized emulsion was determined with a protocol adapted from Christie and previously described.[28,35] The molar amounts of CD and volatile compounds (propanal and hexanal) were measured in a previous work.[23] Results were expressed in mmol per kg of emulsion (mmol kg^{-1} emulsion).

Experimental Design and Data Treatment. For each emulsifier and incubation condition, two emulsions were prepared independently. At each aging time, fluorescence spectra were collected in triplicate using three aliquots of each sample. Protein-bound carbonyls were measured with three test tubes and one control tube. The results correspond to the mean ± standard deviation calculated from the collection of individual data obtained in two emulsions prepared independently.

To test if the proteins issued from the fresh or incubated emulsions and their creamed and aqueous phases had the same protein solubility and amounts of carbonyls, nonparametric Krusdal–Wallis tests were performed for each protein. When a significant effect was found ($p < 0.05$), a multiple range Newman–Keuls posthoc test was performed. The significance level was $p < 0.05$. Statistical analyses were performed with Statgraphics Plus 5.1 software (StatPoint Technologies, Warranton, USA).

Curves of Trp fluorescence at 334 nm were adjusted from the collection of individual data with a modified-Gompertz model. The applied equation was as follows:

$$Y_1 = y_0 - (y_0 - A_1) \exp\left(-\exp\left(1 + \frac{\mu_{Trp}\exp(1) \times (L_{Trp} - t)}{y_0 - A_1}\right)\right) \quad (1)$$

where Y_1 is the intensity of the signal of protein fluorescence at 334 nm normalized with the t_0 intensity (I/I_0), y_0 is the initial value of the normalized signal, namely $y_0 = 1$, t is the incubation time (h), A_1 is the threshold value of the normalized signal, μ_{Trp} is the rate of decrease of the normalized signal (h^{-1}), and L_{Trp} is the lag phase (h).

A nonlinear regression was applied to estimate L and μ with the Marquardt algorithm. The value of A_1 was comprised between 0.10 and 0.30. The initial estimates of L_{Trp} and μ_{Trp} were 0.1 and 0.1, respectively. Asymptote confidence intervals for L and μ values were calculated with a 95% significance level. These calculations were

performed with Statgraphics Plus 5.1 software (StatPoint Technologies, Warranton, USA).

The incubation time when half the minimum normalized intensity of protein fluorescence ($t_{1/2, Trp}$, h) was calculated from eq 2 as follows:

$$t_{1/2,Trp} = L_{Trp} - \frac{\left(\ln\left(-\ln\frac{y_0 - (A_1/2)}{y_0 - A_1}\right) - 1\right) \times (y_0 - A_1)}{\mu_{Trp} \times \exp(1)} \quad (2)$$

■ RESULTS

Fluorescence of Trp and Oxygen Uptake in the Protein-Stabilized Emulsions. In the headspace of BLG, BCN, and BSA solutions (6 g L^{-1}), the oxygen concentration measured for 72 h at 25 °C in the presence of FeSO$_4$/EDTA remained constant. In contrast, the oxygen concentration in the headspace of the emulsions decreased, the kinetics being different according to the incubation conditions.[23,27] Concomitantly, fluorescence spectra of the three protein-stabilized emulsions were recorded for an excitation wavelength of 290 nm at different times of incubation in the presence of FeSO$_4$/EDTA. The spectra at t_0, $t = 24$ h and $t = 48$ are presented in Figure 1A–C. In all emulsions, Trp fluorescence decreased sharply with time. At $t = 48$ h, the recorded signal did not present any more peak in the region of Trp fluorescence (Figure 1A–C). We reported the intensity of the emission at 334 nm versus time and the curves of oxygen uptake recalculated from the kinetic parameters for the emulsions stored at either 33 °C without catalyst or at 25 °C with FeSO$_4$/EDTA (Figures 1D–F). The figures associated to the other conditions (25 °C in the presence of metmyoglobin, FeCl$_3$/sodium ascorbate, or AAPH) are shown in Supporting Information. The estimated lag phases (L_{Trp}) and rates of decrease of the protein fluorescence (μ_{Trp}) were compared to the equivalent parameters obtained from oxygen uptake: L_{O2} and μ_{O2} (Table 2).

In the BLG-stabilized emulsions (Figure 1D), the Trp fluorescence decreased rapidly from the beginning of the incubation period, as highlighted by estimated L_{Trp} values close to zero (Table 2). Then the intensity of the signal reached a plateau corresponding to the background intensity. Half this threshold value ($t_{1/2, Trp}$) was reached after 6.5 or 3.0 h, depending on the incubation conditions. Oxygen uptake also occurred rapidly whatever the incubation conditions and reached a plateau after 30 h.[23] The lag phases estimated for

oxygen uptake (L_{O_2}) were rather short (3.3 and 2.3 h) yet longer than those estimated for the curves of Trp fluorescence (0.2 and ~0 h).

In the BCN-stabilized emulsions (Figure 1E), the Trp fluorescence decreased as well rapidly from the beginning of the incubation period, then reached a plateau corresponding to the maximum extinction of Trp fluorescence. Less than 10 h were necessary to reach half the minimum intensity of Trp fluorescence (Table 2). The estimated lag phases before the decrease of Trp fluorescence were short (1.0 and 3.2 h). Longer lag phases (15.0 and 12.4 h) were observed for oxygen uptake in both incubation conditions.

In the BSA-stabilized emulsions (Figure 1F), the decrease of Trp fluorescence started from the beginning of the incubation period at 25 °C in the presence of FeSO$_4$/EDTA ($L_{Trp} \sim 0$), whereas a 40.3 h lag phase was estimated when incubation was performed at 33 °C (Table 2). Lag phases for oxygen uptake in each incubation conditions were 14.0 and 44.4 h, respectively.[23] They were thus higher than the corresponding lag phases estimated for Trp fluorescence.

When an excess of protein was added in the aqueous phase of BLG- or BCN-stabilized emulsions (Figure 2), no lag phase was still detected for Trp fluorescence (Table 2) but the decrease of Trp fluorescence was slowed down. Accordingly, the rates of decrease of Trp fluorescence (μ_{Trp}) were markedly lower (3.7 × 10^{-2} h^{-1} instead of 6.8 × 10^{-2} h^{-1} for the BLG-stabilized emulsions, and 1.3 × 10^{-2} h^{-1} instead of 5.8 × 10^{-2} h^{-1} for the BCN-stabilized emulsions). Oxygen uptake was as well delayed in the presence of an excess of proteins. This led to longer lag phases: 9.9 h instead of 3.3 h for the BLG-stabilized emulsions and 44.4 h instead of 15.0 h for the BCN-stabilized emulsions. It was markedly slowed down when excess BLG was added to the emulsion (6.1 instead of 3.9 mmol 0$_2$ kg^{-1} oil h^{-1}).

Finally, Figures 1 and 2 and Supporting Information showed a good global time-coincidence between oxygen uptake and decrease of Trp fluorescence. When the decrease of Trp fluorescence started early in the emulsions, oxygen uptake started early as well. On the opposite, when the decrease of Trp fluorescence did not start immediately, a rather long lag phase was observed for oxygen uptake. This relationship is highlighted in Figure 3A, which shows a linear relationship (R^2 = 0.84) between the estimated time to reach half the baseline fluorescence signal ($t_{1/2, Trp}$) and the estimated time to reach half the maximum oxygen uptake ($t_{1/2, O_2}$) whatever the incubation conditions. These figures also evidence that $t_{1/2, Trp}$ was always shorter than $t_{1/2, O_2}$. In agreement, the lag phases estimated for Trp fluorescence were always shorter than those estimated for oxygen uptake, showing that protein fluorescence decreased always earlier than headspace oxygen (Figure 3B and Table 2).

Modifications of Adsorbed and Unadsorbed Proteins in the Oxidizing Emulsions. To compare the modifications undergone by proteins either adsorbed on the droplet surface or unadsorbed in the aqueous phase, we collected and analyzed these two fractions after centrifugation of emulsion samples at 0, 24, and 48 h of incubation.

Tryptophan Fluorescence. Figure 4 represents the fluorescence spectra acquired for the aqueous and creamed phases of BLG-, BCN- and BSA-stabilized emulsions at pH 6.7 without excess of protein, at t_0 or after 24 or 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. In accordance with the distribution of proteins within emulsions (Table 1), the initial
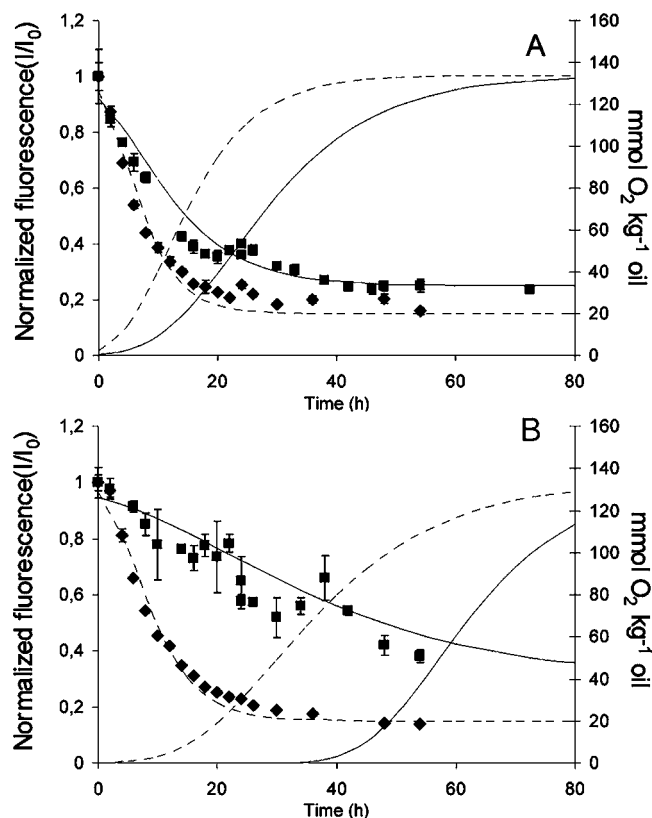
Figure 2. Effect of excess of proteins in the aqueous phase of emulsions on the normalized Trp fluorescence ($\lambda_{excitation}$ = 290 nm, $\lambda_{emission}$ = 334 nm) (curves with symbols) and oxygen uptake (curves without symbols). Emulsions were stabilized with (A) BLG or (B) BCN at pH 6.7, without ($\blacklozenge$, dotted lines) or with ($\blacksquare$, continuous lines) excess of protein in the aqueous phase, and incubated at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. The curves correspond to the modified Gompertz equation calculated with the estimated L and μ parameters. Errors bars represent standards deviations (n = 3 to 6 for fluorescence data; n = 6 to 12 for oxygen uptake data). Abbreviations: BLG = β-lactoglobulin; BCN = β-casein; BSA = bovine serum albumin; EDTA = ethylene diamine tetraacetic acid; Trp = tryptophan; L = lag phase (h); μ = reaction rate (h^{-1}).

fluorescence intensity was higher in the creamed phase samples than in the aqueous phase samples, especially in the BCN-stabilized emulsion, where the BCN concentration in the aqueous phase was very low (0.45 g L^{-1}, Table 1). In all creamed phases, the Trp fluorescence decreased dramatically upon incubation. After 48 h of incubation, protein emission was not detected in the creamed phases; only the creamed phases of BCN-stabilized emulsions presented residual protein emission at 24 h (Figure 4). In the aqueous phase of the BLG- and BSA-stabilized emulsions, the decrease of protein fluorescence was less marked; protein fluorescence emission remained significant after 48 h of incubation with a distinguishable fluorescence maximum located around 334 nm.

Protein Solubility. The solubility of proteins extracted from whole emulsions, aqueous phase, or creamed phase of BLG-, BCN-, and BSA-stabilized emulsions at pH 6.7, without excess of protein, was investigated by determining the concentration of soluble proteins in guanidine chloride 6 M at t_0, after 24 and 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM (Figure 5). In the BLG- and BSA-stabilized emulsions and in their creamed phases, the solubility of proteins decreased significantly ($p < 0.05$) during the incubation period. After 48 h
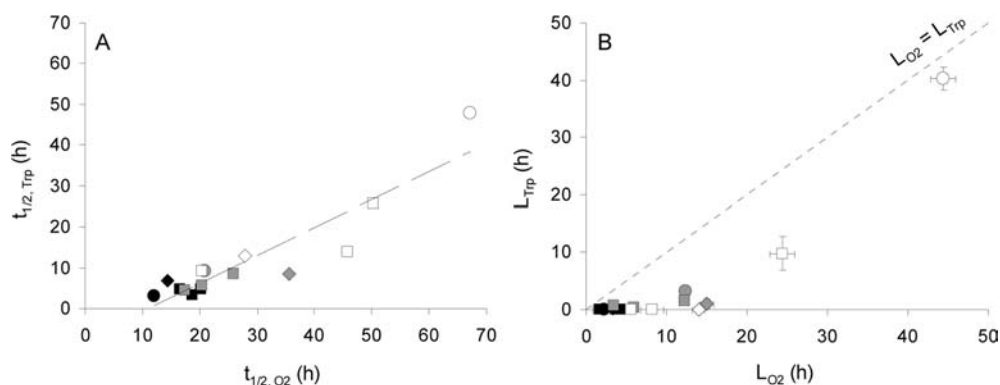
Figure 3. Relationship between the estimated parameters for Trp fluorescence and oxygen uptake: (A) $t_{1/2, \text{Trp}}$ as a function of $t_{1/2, \text{O2}}$; (B) L_{Trp} as a function of L_{O2}. Data represent the parameters estimated during incubation of emulsions stabilized with BLG (black symbols), BCN (gray symbols), or BSA (white symbols), at 33 °C without oxidation initiator (circles) or at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM (diamonds) or with the other oxidation initiators (squares). In (A), the large-dotted line represents the linear model applied to the whole data set (R^2 = 0.84). Errors bars represent the asymptote confidence intervals calculated for L values with a 95% significance level. In (B), the small-dotted line represents $L_{\text{Trp}} = L_{\text{O2}}$. When not shown, error bars are within data points. Abbreviations: BLG = β-lactoglobulin; BCN = β-casein; BSA = bovine serum albumin; EDTA = ethylene diamine tetraacetic acid; Trp = tryptophan; $t_{1/2}$ = incubation time when half the maximum oxygen uptake of fluorescence decrease are reached (h); L = lag phase (h); μ = reaction rate (h^{-1}).

of incubation, the solubility of proteins extracted from the creamed phases reached less than 10% of the t_0 value. In the BCN-stabilized emulsion, the solubility of the proteins from whole emulsion or creamed phase was not modified after 24 h of incubation ($p > 0.05$) but was significantly reduced after 48 h of incubation. In contrast, for all emulsions, the solubility of the proteins of the aqueous phases was not significantly affected whatever the incubation time ($p > 0.05$).

Protein Aggregation: SDS PAGE. To investigate the modifications undergone by the proteins during incubation of emulsions, proteins from whole emulsions, aqueous phase, or creamed phase of BLG-, BCN-, and BSA-stabilized emulsions at pH 6.7, without excess of protein, were analyzed by SDS-PAGE at t_0, after 24 or 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM (Figure 6). The possible involvement of disulfide bonds was examined by performing SDS-PAGE in either nonreducing or reducing conditions.

The lanes for native BLG presented a main band at around 18 kDa, which corresponds to the monomer form of the protein, and a less intense band at around 37 kDa, which corresponds to its dimer form. In the aqueous phase of BLG-stabilized emulsions, no modifications in the SDS-PAGE profiles occurred along the incubation period. In contrast, for the whole emulsion and creamed phase, the intensity of the bands corresponding to the monomers of protein decreased markedly along the incubation period in both nonreducing and reducing conditions. Concomitantly, high molecular weight fractions corresponding to aggregates ranging from around 30 to more than 100 kDa appeared. The decreasing intensity of the BLG monomer band was more marked in the creamed phase than in the whole emulsion samples due to the contribution of the aqueous phase. In the nonreducing conditions, no more monomer band was detectable for the creamed phase sample after 48 h of incubation (only 3% of the t_0 band intensity as estimated by densitometric analysis). When the electrophoresis of the proteins from the creamed phase was performed in reducing conditions, high molecular weight fractions remained present but the intensity of the BLG monomer band was partially recovered, especially after 48 h of incubation (about 35% of the initial intensity).

The lanes for native BCN presented a main band at around 24 kDa corresponding to the monomer form, two thin bands with a slightly lower molecular weight, and a low intense band at around 50 kDa corresponding to the dimer form. For the aqueous phase of BCN-stabilized emulsions, the intensity of the main band at around 24 kDa increased by almost two times at t = 24 h and t = 48 h of incubation as compared to t_0. This is in accordance with the increase of the concentration of unadsorbed BCN during the aging of the emulsions (Table 1).[28] For the whole emulsion and creamed phase samples, the intensity of the main band decreased by around 50%, thus in a lesser extent than for BLG. This agrees with the lower BLG solubility in the creamed phase after 48 h of incubation as compared with BCN (Figure 5). The development of higher molecular weight fractions occurred, as highlighted by the emergence of diffuse bands attributed to aggregates. No difference was found between the nonreducing and reducing conditions because BCN does not contain any cysteyl residue nor disulfide bonds.

Finally, the lanes for native BSA comprised a main band at around 66 kDa corresponding to the monomer form, several bands of higher molecular weight corresponding either to traces of BSA aggregates or to contaminating proteins (immunoglobulins), and a few thin bands with a slightly lower molecular weight. In the aqueous phase of BSA-stabilized emulsions, no modification in the profile was observed along the incubation period. In the same time, a huge decrease of the intensity of the monomer band was observed in the whole emulsion and creamed phase samples (50% and 10% of residual intensity as compared to t_0, respectively). In the latter sample, the band was not longer detectable after 48 h of incubation when electrophoresis was performed in nonreducing conditions (only 2% of residual intensity). The monomer band was slightly recovered when the reducing conditions were applied (8% of residual intensity). This suggests that the protein aggregation induced during the incubation period was weakly due to the formation of disulfide bonds. Contrary to the observations in BLG and BCN profiles, higher molecular weight fractions were not detected in the incubated whole emulsion and creamed phase samples of BSA. BSA aggregation
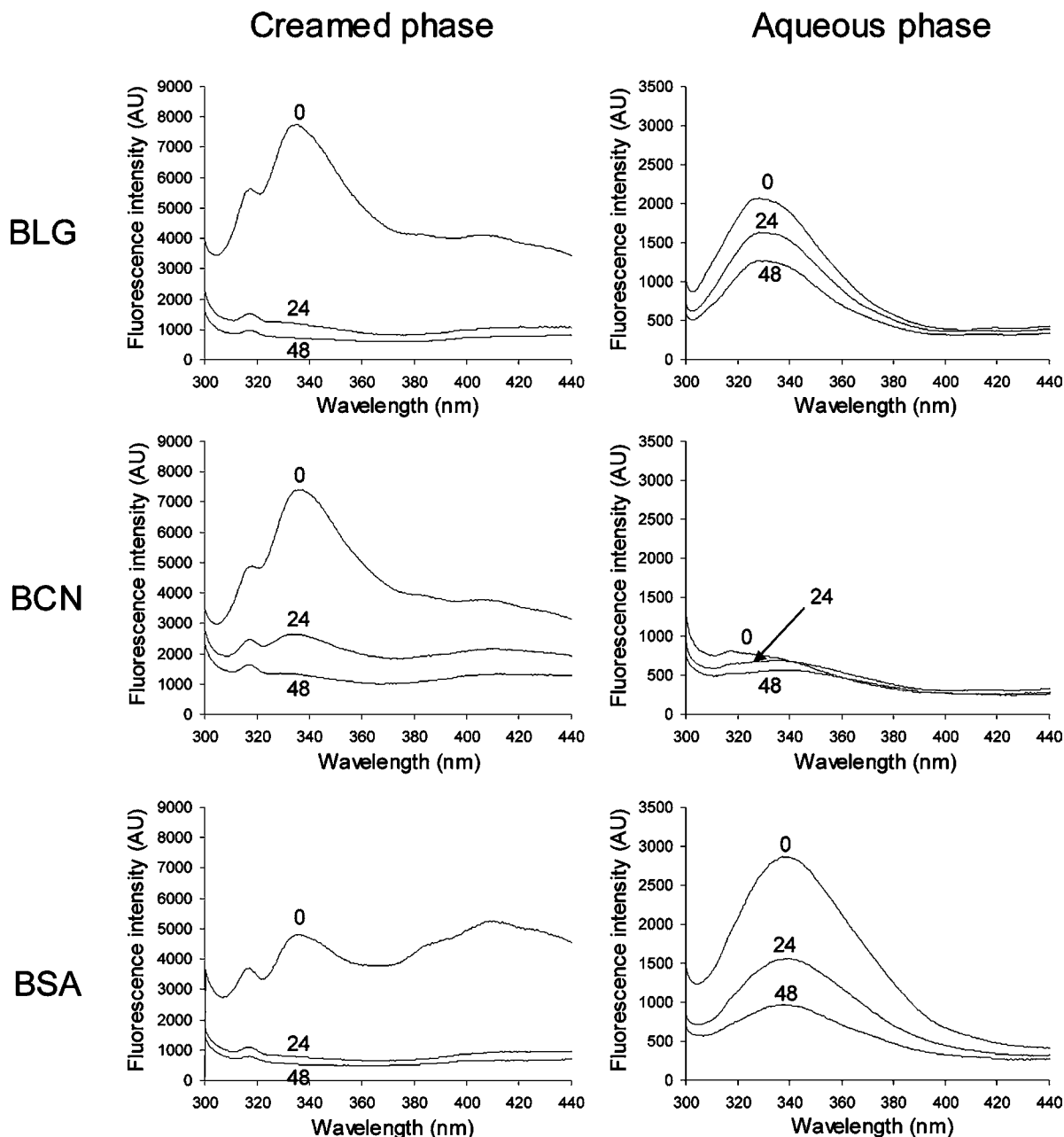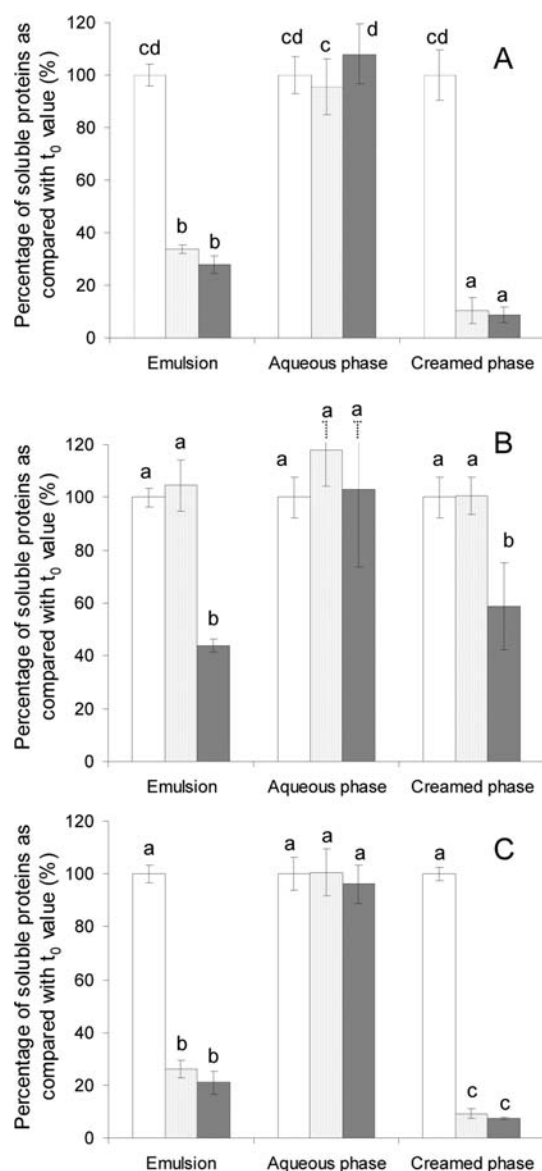
Figure 4. Fluorescence spectra ($\lambda_{excitation}$ = 290 nm) of the creamed phase or aqueous phase of emulsions stabilized by BLG, BCN, or BSA without excess of protein, freshly prepared (t = 0) or after 24 or 48 h of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM.

may lead to very high molecular weight polymers, which could not penetrate into the gel.[18,36]

To summarize, the intensity of the bands corresponding to the protein monomers did not decrease along the incubation period in the aqueous phases. On the opposite, the intensity of the bands corresponding to the protein monomers decreased in the creamed phases, in both nonreducing and reducing conditions. These observations are in accordance with the results of protein solubility presented above, and thus confirm that in the oxidizing emulsions, the proteins present in the aqueous phase were less modified than the adsorbed proteins.

Formation of Protein-Bound Carbonyls. The formation of carbonyls on the proteins extracted from whole emulsions, aqueous phase, or creamed phase of BLG-, BCN-, and BSA-stabilized emulsions at pH 6.7, without excess of protein, was investigated at t_0 after 24 or 48 h of incubation at 25 °C with

FeSO$_4$/EDTA 1/1 M/M 200 μM (Figure 7). For the three proteins, the formation of carbonyls was significantly higher in the creamed phases than in the aqueous phases ($p < 0.05$). The amounts of protein-bound carbonyls formed in the whole emulsions were accordingly intermediate. In the BLG-stabilized emulsions, around 1 and 10 μmol carbonyls per g of proteins were detected at t_0 in the aqueous and creamed phases, respectively. These amounts increased significantly during the incubation period ($p < 0.05$), finally reaching 12 and 70 μmol g^{-1} of soluble proteins in the aqueous and creamed phases, respectively. In the BCN-stabilized emulsions, very low initial amounts of carbonyls were detected (lower than 2 μmol g^{-1} proteins in emulsion, creamed and aqueous phases). The carbonyl formation had increased significantly ($p < 0.05$) after 24 h of incubation in emulsion and creamed phase and continued to increase thereafter. After 48 h of incubation, it

Figure 5. Solubility in GuCl 6 M of proteins extracted from whole emulsions, creamed phase, or aqueous phase of emulsions stabilized with (A) BLG, (B) BCN, or (C) BSA, without excess of protein, in freshly prepared emulsions (white bars) or after 24 h (dotted bars) or 48 h (gray bars) of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. Mean values (n = 5 to 8) of percentages of soluble proteins reported to the t_0 value (%). Errors bars represent standard deviations. In the same figure, different letters indicate significant difference ($p <$ 0.05) .

reached almost 50 μmol g^{-1} of soluble proteins in the creamed phase. In contrast, the carbonyl content in the aqueous phase did not increase significantly ($p >$ 0.05). Finally, in the BSA-stabilized emulsions, low initial amounts of carbonyls were detected, lower than 4 μmol g^{-1} of proteins in emulsion, aqueous phase, and creamed phase. The amount of carbonyls increased significantly ($p <$ 0.05) after the first 24 h of incubation in the emulsion and the creamed phase. This increase was very marked in the creamed phase in which the amount of carbonyls leveled off after 24 h of incubation ($p >$ 0.05) and stabilized at around 50 μmol g^{-1} soluble proteins. The formation of carbonyls in the aqueous phase was more gradual, suggesting a slower degradation of the unadsorbed

proteins as compared with the adsorbed ones. After 48 h of incubation, the amount of protein-bound carbonyls (20 μmol g^{-1} soluble proteins) in the aqueous phase of BSA-stabilized emulsions was higher than in the aqueous phase of the BLG- and BCN-stabilized emulsions (around 12 and 6 μmol g^{-1} soluble proteins, respectively).

Molar Balance of Substrates and Reaction Products in Oxidized Emulsions. Molar balances of several substrates and products of lipid oxidation and protein modifications were calculated to provide a quantitative view of the reactions arising in the oxidizing emulsions (Table 3). Calculations were based on the amount of PUFA in freshly stripped rapeseed oil or extracted from an oxidized BLG-stabilized emulsion and the amounts of consumed oxygen, CD, and selected volatile compounds arising from PUFA oxidation and protein-bound carbonyls.[23] In the oxidized emulsion at after 30 h of incubation (25 °C; FeSO$_4$/EDTA, 1/1, M/M, 200 μM), approximately 8% of C18:2 n-6 and 17% of C18:3 n-3 were lost. The amount of consumed oxygen was around 39 mmol kg^{-1} emulsion, whereas the total amount of oxidized PUFA was around to 28 mmol kg^{-1} (Table 3). CD detected at the same incubation time (22 $\pm$ 2.6 mmol kg^{-1}) were about half of consumed oxygen. The amounts of propanal and hexanal (0.137 and 0.022 mmol kg^{-1} emulsion, respectively) were 160 and 200-fold lower than the amounts of lost linoleic and linolenic acids (14 mmol kg^{-1} emulsion). Finally, the amount of protein-bound carbonyls was low in comparison with the amount of consumed oxygen (31 μmol kg^{-1} versus 39 mmol kg^{-1}) but also in comparison of detected primary (conjugated dienes) and secondary (volatile compounds) products of oxidation.

■ DISCUSSION

The two main chemical phenomena arising in the oxidizing protein-stabilized emulsions are lipid peroxidation[1,36] and protein chemical modifications, including protein oxidation.[8,18,37] In addition, oxidation initiators such as Fe/EDTA are involved in the initial free radical reactions and can participate in other reactions such as decomposition of hydroperoxides or Fe^{2+}/Fe^{3+} redox cycling reactions.[38,39] These three reaction pathways could contribute to oxygen uptake in our emulsions. First, protein solutions incubated in the same conditions than the emulsions did not present any measurable oxygen consumption. Second, the molar balance in the BLG-stabilized emulsion (Table 3) showed that the molar amounts of consumed oxygen (around 39 mmol kg^{-1}), lost PUFA (28 mmol kg^{-1}), and measured CD (22 mmol kg^{-1}) were in the same order of magnitude and 1000 times higher than the molar amounts of protein carbonyls produced in the emulsions. In agreement with previous work showing that oxygen uptake in protein-stabilized emulsions containing polyunsaturated lipids was mainly linked to lipid oxidation,[23,27,29,30] we deduced that protein modifications slightly contributed to oxygen consumption in our systems whereas lipid oxidation was the main contributor. The twice lower amount of CD detected as compared to oxygen uptake can be explained first by the simultaneous formation and decomposition of hydroperoxides, assessed by the rise of volatile compounds and second by the formation of lipid derivatives not presenting the dienic structure (i.e., hydroperoxides from oleic acid but also from nonconjugated derivatives from polyunsaturated fatty acids) and therefore not detected by the UV method.
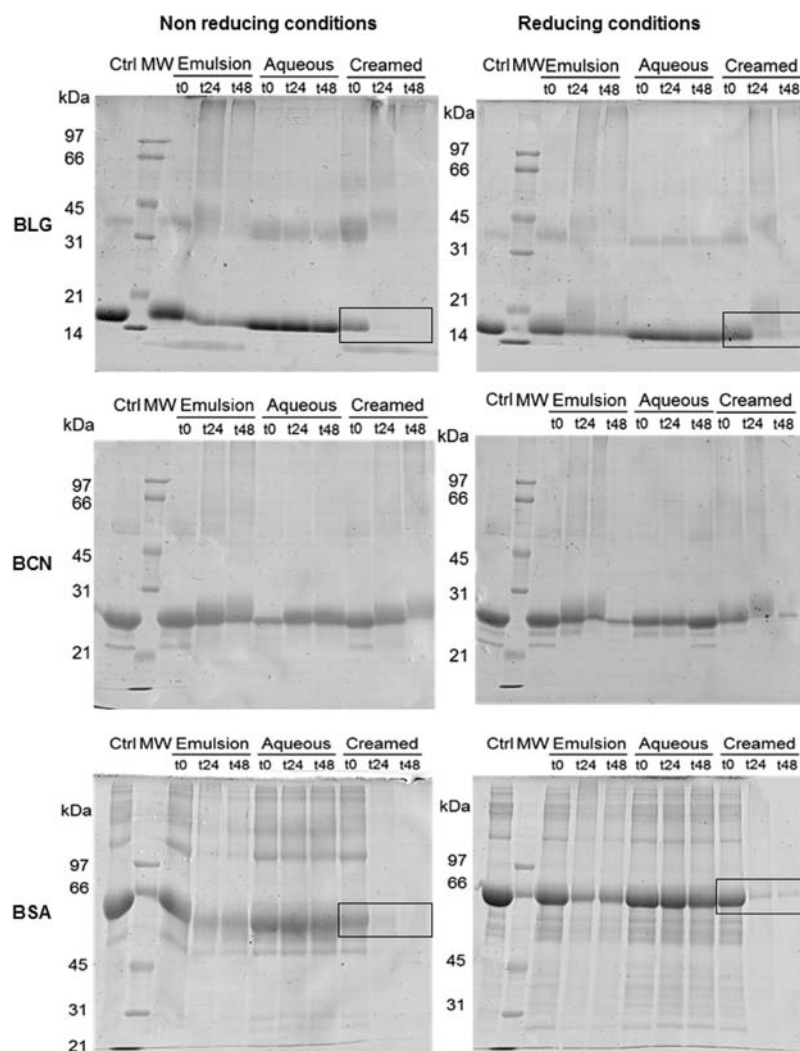
Figure 6. SDS-PAGE profiles of proteins extracted from whole emulsions (lanes 3, 4, 5: t_0, $t = 24$ h, $t = 48$ h), aqueous phase (lanes 6, 7, 8: t_0, $t = 24$ h, $t = 48$ h) or creamed phase (lanes 9, 10, 11: t_0, $t = 24$ h, $t = 48$ h) of emulsions, without excess of protein, during the incubation of BLG-, BCN-, or BSA-stabilized emulsions at 25 °C with $FeSO_4$/EDTA 1/1 M/M 200 μM. In each gel, lane 1 corresponds to the native protein and lane 2 to the molecular weight marker.

Protein Modifications As an Early Event in the Oxidative Chain Reactions. Parallel to the evaluation of lipid oxidation in the emulsions through oxygen uptake, presented in our previous work,[23,27] the modifications undergone by the proteins were assessed by the evolution of Trp fluorescence measured in situ by front-surface fluorescence. A decrease of tryptophanyl fluorescence in a complex system can result from several mechanisms such as changes in the environment of tryptophanyl residues due to modifications of the protein conformation, static quenching due to interactions with small molecules, chemical modification on the Trp indol ring through free radical reactions, or inner filter effects due to changes in the light scattering properties of the sample.[32,40] In the oxidizing emulsions, the decrease of Trp fluorescence cannot be clearly attributed to one or the other mechanism. However, the method is recognized as a global indicator of protein degradation in oxidizing systems.[12,16,21,22,41−43] In these papers using model systems or real foods, protein modifications were timely linked with lipid oxidation. In our emulsions stabilized with BLG, BCN, or BSA and incubated in various conditions, a linear relationship between $t_{1/2,Trp}$ and $t_{1/2,O2}$ was shown (Figure 3A). Additionally, the comparison of the lag

phases L_{Trp} and L_{O2} demonstrates that the decrease of Trp fluorescence always preceded oxygen uptake (Figure 3B, Table 2). As the difference between L_{Trp} and L_{O2} is not constant, it cannot be attributed to only the detection thresholds of the oxygen uptake method and front-surface fluorescence. We assume that these different lag phases result from different rates for the initiation reactions involved in both protein modifications and lipid oxidation, these rates depending on the emulsifier-oxidation initiator couple.[30] In a previous work, we measured the formation of conjugated dienes in the same protein-stabilized emulsions incubated in the presence of Fe/EDTA:[23] the herein estimated lag phases for CD formation were also higher than L_{Trp}, showing that CD formation also followed the decrease of protein fluorescence. To conclude, our data indicate that protein modifications, as revealed by the decrease of protein fluorescence, started before the radical attack of the lipid phase and the propagation step of lipid oxidation, evaluated either by O_2 uptake or from CD measurements.

The reaction sequence between lipid and protein oxidation in complex multiphase matrices is currently debated in the literature. Our results are in agreement with recent work on
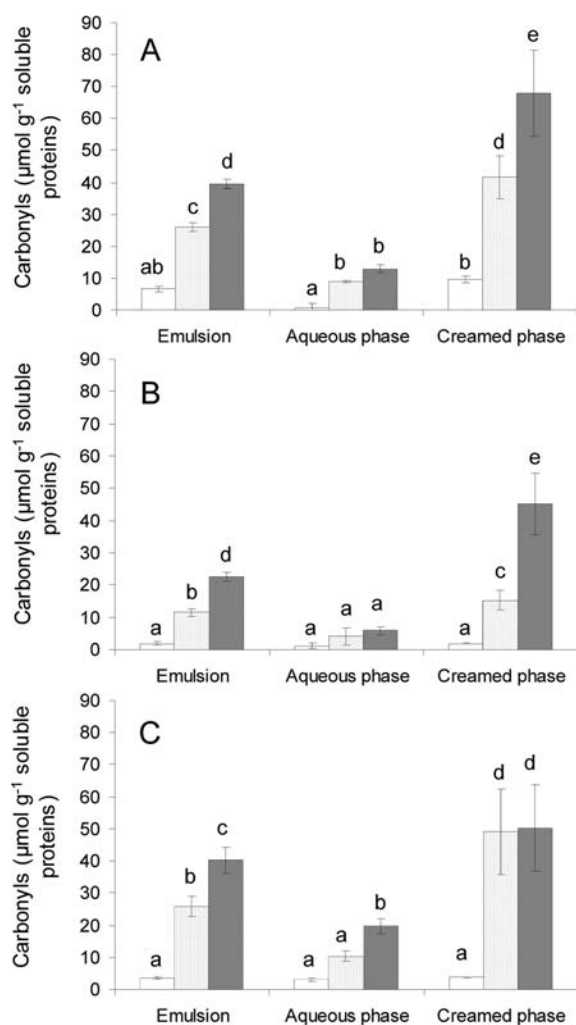
Figure 7. Protein-bound carbonyls in whole emulsions, creamed phase, or aqueous phase of emulsions stabilized with (A) BLG, (B) BCN, or (C) BSA, without excess of protein, in freshly prepared emulsions (white bars) or after 24 h (dotted bars) or 48 h (gray bars) of incubation at 25 °C with FeSO$_4$/EDTA 1/1 M/M 200 μM. Results are mean values of six replicates ($n = 6$) except $n = 4$ for the creamed phases of BCN/t48h and BSA/t24h. They are expressed in μmol carbonyls per g GuCl 6 M soluble proteins. Errors bars represent standard deviations ($n = 6$). For one protein, different letters indicate significant difference ($p < 0.05$).

model emulsions showing that BLG is present in the aqueous phase or myofibrillar proteins oxidized prior to the detection of

lipid oxidation.[16,21,43] These early modifications of proteins do not exclude a transfer of oxidative reactions from lipids to proteins in the latest stages of oxidation when lipid oxidation propagates, as assumed by several authors.[8,12,15,17,19]

Adsorbed Proteins As the Main Target of Protein Modifications in Oxidizing Emulsions. The fluorescence intensities measured in the whole emulsions result from the signals emitted by both adsorbed and unadsorbed proteins. Our emulsions were formulated to avoid the presence of a large excess of proteins in the aqueous phase. Thus, 90% to 70% of proteins were actually located at the interface (Table 1). In these emulsions, the decrease of protein fluorescence (Figures 1, 2) was therefore mostly related to the adsorbed proteins. To distinguish nevertheless between these two contributions, Trp fluorescence spectra were acquired on the creamed and aqueous phases of the emulsions. The results confirm that during emulsion incubation, the decrease of protein fluorescence should be mainly attributed to the adsorbed proteins whereas it remained moderate in the unadsorbed proteins (Figure 4). Our results are in accordance with Rampon et al., who demonstrated that decreasing the size of oil droplets in BSA-stabilized emulsions, and thus increasing the total interfacial area and the proportion of adsorbed BSA, led to a faster and more marked decrease of Trp fluorescence.[22] They concluded that BSA was more modified when located at the oil droplet surface than unadsorbed in the aqueous phase.

The strong decrease of protein solubility in the creamed phases of oxidizing emulsions corroborates the higher modifications of the adsorbed proteins in comparison to the proteins in the aqueous phase. The loss of solubility of interfacial proteins during the incubation of emulsions is presumably related to the aggregation and covalent modifications of proteins. BLG and BSA are prone to aggregation in oxidizing conditions.[18,19,44] Protein cross-linking induced by oxidation can occur via either cysteyl residues forming disulfide bonds or tyrosyl residues leading to dityrosyl cross-links or quinone structures, which can themselves form complex cross-link structures by reacting with other amino acids (lysine, cysteine, proline).[12,20,45] The minor involvement of disulfide bonds in the aggregation of adsorbed BLG and BSA was highlighted from the results of SDS-PAGE (Figure 6). BCN does not contain any free cysteyl residue, and no difference between the SDS-PAGE profiles in reducing and nonreducing conditions was thus observed. BLG, BSA, and BCN contain tyrosyl residues (4, 20, and 4, respectively). This makes possible the formation of dityrosine, widely mentioned as a factor involved in aggregation of proteins in the presence of oxygen

Table 3. Molar Balance of Oxidation Substrates and Products in BLG-Stabilized Emulsion after 30 h of Incubation (25 °C; FeSO$_4$/EDTA, 1/1, M/M, 200 μM)[a]

	total amount of PUFA[b]		oxidized PUFA[b]	consumed O$_2$[b,c]	CD[b]	volatile compounds[b]	protein-bound carbonyls[b,d]
time (h)	0	30	30	30	30	30	24
C18:2 n-6	168.1 ± 0.5	153.9 ± 1.4	14			0.022[e] (hexanal)	
C18:3 n-3	81.2 ± 0.3	67.1 ± 0.8	14			0.137[e] (propanal)	
total	249	221	28	39.0 ± 1.5	22.0 ± 2.6	0.159	0.031 ± 0.002

[a]Experimental values correspond to the means of at least triplicate determinations ± standard deviation, otherwise stated. Abbreviations: BLG = β-lactoglobulin; CD = conjugated dienes; EDTA = ethylene diamine tetraacetic acid; PUFA = polyunsaturated fatty acids. [b]Expressed as mmol kg^{-1} emulsion. [c]From Berton et al.[27] [d]To evaluate the molar concentration of BLG-bound carbonyls in the emulsions after 24 h of incubation, the unsoluble fraction of the protein was considered to present similar amount of carbonyls than the soluble fraction (34% of total BLG). [e]Mean of two independent measurements.

reactive species[10,12,42,45] or radicals arising from oxidizing lipids.[8,15,46] Proteins can also undergo covalent modifications induced by aldehydes arising from lipid oxidation.[8] 4-Hydroxynonenal (4-HNE) and 4-oxononenal were demonstrated to form covalent adducts with nucleophilic amino acids (cysteine, histidine, lysine, and arginine).[47] Such covalent interactions were highlighted between milk proteins and 2-hexenal or in a lesser extent hexanal, involving lysyl and histidyl residues.[48] Mass spectroscopy analysis of BCN extracted from an oxidized emulsion revealed an increase of approximately 300 Da in the protein mass, which was attributed to the addition of unsaturated aldehydes arising from oil oxidation on BCN.[49]

Ultimately, the favored oxidation of adsorbed proteins as compared to unadsorbed ones was clearly highlighted from the measurement of protein-bound carbonyls (Figure 7). Carbonylation is recognized as a major modification in oxidized proteins.[34,50,51] Carbonyl derivatives can be formed by direct oxidative attack of prolyl, arginyl, lysyl, and threonyl residues, especially in the presence of metal ions. They can also arise from oxidative cleavage of the peptide backbone via the α-amidation pathway or through secondary reactions between lipid oxidation products and lysyl, cysteyl, and histidyl residues.[51] In our work, the major involved pathways for protein carbonyl formation are presumably the reactions involving oxidizing lipids. This is in agreement with the results of Refsgaard et al., showing that the presence of PUFA stimulated the formation of BSA-bound carbonyls.[52] The unadsorbed proteins, although directly in contact with the reactive oxygen species in the aqueous phase, were by far less prone to carbonylation than the adsorbed proteins. As in the creamed phases, protein-bound carbonyls could be quantified only in proteins soluble in GuCl that represented less than half of the total proteins (Figure 5), the amounts of protein carbonyls in these phases are even underevaluated. In contrast, in situ methods such as front-surface fluorescence provide information about the whole protein fraction contained in the samples.

The role of unadsorbed proteins in oxidizing emulsions is intricate. On the one hand, unadsorbed proteins were clearly less oxidized and modified than adsorbed proteins, which was demonstrated through all the tested methods. On the other hand, the addition of excess proteins in the aqueous phase of emulsions tremendously delayed and sometimes slowed down lipid oxidation, which implies that unadsorbed proteins are able to delay the access of prooxidant species toward the lipid core of oil droplets. Indeed, unadsorbed proteins can scavenge free radicals and/or chelate metal ions, as mentioned earlier.[25,30,53,54] We assume that unadsorbed proteins exert their antioxidant role mainly during the initiation step of oxidation, during which low amounts of free radicals are involved. This would explain why unadsorbed proteins undergo low damage (unchanged solubility, no aggregation, low levels of protein carbonyls, significant decrease of protein fluorescence but to a lower extent than that in the creamed phase) while delaying efficiently lipid oxidation. Then, once the propagation step is engaged in the lipid phase, larger amounts of reactive species are produced and the reactions at the interface between proteins and oxidizing lipids become quantitative, inducing protein modifications (loss of solubility, aggregation, formation of protein carbonyl, total extinction of protein fluorescence).

This study brings both kinetic and spatial insights regarding protein degradations in lipid-based multiphase systems. First, a strong time relationship was found between lipid oxidation and protein modifications. The kinetic parameters demonstrated that protein modifications, indicated by a decrease of protein fluorescence, started however prior to lipid oxidation. Second, the location of proteins within the emulsions appears as a crucial factor controlling the extent of their oxidative modifications. The proteins adsorbed on the oil droplet surface were strongly modified along the incubation of emulsions, whereas the unadsorbed proteins, which are involved in delaying lipid oxidation through the quenching of aqueous free radicals and/or binding of oxidation initiators, underwent no substantial degradations.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Fluorescence decrease and oxygen uptake in the emulsions incubated at 25 °C in the presence of metmyoglobin, $FeCl_3$/sodium ascorbate, or AAPH. This material is available free of charge via the Internet at http://pubs.acs.org.

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