Interface Characterization and Aging of Bovine Serum Albumin Stabilized Oil-in-Water Emulsions As Revealed by Front-Surface Fluorescence

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This paper is devoted to the application of front-surface fluorescence to the study of aging and oxidation of oil-in-water emulsions. Emulsions with two oil droplet sizes were stabilized with bovine serum albumin (BSA) and stored at 37 or 47 °C. Lipid oxidation was demonstrated by measurement of hydroperoxides and headspace pentane. Front-surface fluorescence spectra (excitation wavelength = 355 nm) revealed gradual formation of oxidized lipid−protein adducts during the 4 weeks of storage. Fluorescence (excitation = 290 nm) of BSA tryptophanyl residues (Trp) declined during the first day of aging and then decreased slightly and linearly. Fourth-derivative Trp spectra exhibited peaks at 316 and 332 nm. Their evolution indicated that the ratio of Trp in hydrophobic environments to total Trp increased in small droplet emulsions. This suggests that, during lipid oxidation, the adsorbed and nonadsorbed protein underwent various degrees of Trp degradations, polymerization, and aggregation. Thus, front-surface fluorescence makes it possible to evaluate, noninvasively, protein modification and lipid oxidation end-products during processing and storage of food emulsions.

Keywords: *Front-surface fluorescence; emulsion; bovine serum albumin; lipid oxidation; interface; tryptophanyl residues; oxidized lipid−protein adducts*

INTRODUCTION

A large number of foods exist in the form of emulsions. Examples include milk, cream, mayonnaise, soups, sauces, and butter. Emulsions are stabilized due to emulsifiers and surfactants that populate the oil−water interface and lower the interfacial tension. Food emulsions generally contain proteins as emulsifiers in addition to smaller molecules such as monoglycerides and phospholipids (*1*). They undergo diverse treatments, including storage in various conditions, before they reach the consumer's plate. These treatments induce emulsion modifications such as protein denaturation, aggregation, or polymerization (*2*, *3*), physical destabilization (*4*), interface aging (*5*, *6*), and chemical reactions including lipid oxidation (*7*, *8*) and oxidized lipid−protein reactions (*5*, *6*). Undesirable off-flavors and potentially toxic reaction compounds may develop that make the products no longer acceptable for human consumption. Characterization of the chemical changes that affect processed emulsions require many complementary methods to simultaneously quantify primary and secondary products of oxidation and protein modification. Most of these methods are destructive and time-consuming.

Fluorescence is the emission of photons from singlet electron-excited states of a molecule. Sensitive, selective, and adaptable, fluorescence spectroscopy has been used in many studies of molecular structures and interactions, in the localization of molecules, and in many types of trace analysis (*9*). Davies (*10*, *11*) monitored tryptophan degradation and bityrosine production upon oxidation by oxygen radicals of a wide variety of proteins. Fluorescence of secondary oxidation products resulting from interactions between lipid oxidation compounds and reactive amino acids of proteins have also been detected (*12*, *13*). Aubourg (*14*) observed the appearance of a progressively red-shifted fluorescence of solvent-extracted lipids as a result of oxidation and damage of fish products.

Fluorescence also provides information about the environment of the tryptophanyl residues of proteins and their interactions with lipids. Generally applied to diluted optically clear samples, this technique can also be carried out using front-surface geometry to allow the analysis of concentrated solutions, suspensions, and solid samples (*15*). Liang (*16*) showed a good correlation between thiobarbituric acid (TBA) value, protein solubility, and front-surface fluorescence intensity for oxidized lipid−protein adducts in dry mixtures of soybean oil and soy proteins stored at 60 °C. This author also studied changes in the fluorescence and peroxide value of stored milk powder (*17*) and soybean flour (*18*). Using this method, conformational changes of bovine serum albumin (BSA) upon its adsorption on the interface in dodecane-in-water emulsions were directly measured (*19*), and the partition of adsorbed to nonadsorbed BSA in emulsions was calculated (*20*).

In this paper, front-surface fluorescence was used to simultaneously study (i) the protein interface, (ii) protein aging, and (iii) the formation of secondary oxidation products during storage of oil-in-water emulsions stabilized by BSA. The effects of oil droplet size and storage temperature were also investigated.

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MATERIALS AND METHODS

Materials and Chemicals. BSA was from Cohn fraction V, the preparation of which is performed under non-denaturing conditions (purity = 95−98%, ref 103703, ICN Biomedicals Inc.). Xylenol orange (ref 152269) was obtained from ICN Biomedicals Inc. Sodium azide and hydrochloric acid (1.09970, Titrisol) were purchased from Merck. Sulfuric acid, ammonium ferrous sulfate, butylated hydroxytoluene (BHT), methanol, 2-propanol, and pentane were from Carlo Erba (analytical quality). Cumene hydroperoxide was obtained from Sigma (ref C-0524). Ultrapure water (18 MΩ) was prepared using a Milli-Q system and used for all aqueous solutions.

Commercial sunflower oil was stripped of tocopherols, mono- and diacylglycerides, and free fatty acids on an alumina column (Alumina N; Akt. I; ref 020287; ICN Biomedicals Inc.). Stripped oil contained 0.5% [mono- + diacyl-]glycerides and virtually no tocopherols or free fatty acids. Its fatty acid composition (total fatty acid basis) was 65.0% linoleic acid, 21.5% oleic acid, 6.4% palmitic acid, and 5.0% stearic acid.

Preparation of BSA Solutions. BSA was gradually dissolved in ultrapure water containing 0.4 g/L sodium azide. Then the pH was adjusted to 4.4 with 4 N hydrochloric acid. The final concentration of BSA solution used to prepare emulsions was 20 g/L. The concentration of control BSA solution was 14 g/L to obtain the same BSA concentration as in emulsions. The latter was stored under the same conditions as the emulsions.

Preparation of Emulsions. Twenty-eight milliliters of a 20 g/L BSA solution and 12 mL of purified sunflower oil were premixed for 30 s at 23000 rpm with a polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) fitted with a 12 mm diameter head. The coarse emulsion was then recirculated for 3 min through a high-pressure valve homogenizer (Stansted Fluid Power, Stansted, U.K.). A one-stage homogenization process at 15 bar was used to obtain "low-pressure emulsions". A two-stage process with valves applying successively 200 and 40 bar provided "high-pressure emulsions". Aliquots (6 mL) of emulsions were distributed in 22.4 mL headspace vials (ref 5182-0837, Hewlett-Packard), sealed with PTFE/silicon septa and aluminum crimp seals. The vials were rotated in the dark at 20 rpm with a test tube rotator (Labinco B.V., Ac Breda, The Netherlands) either at 37 ± 2 °C or at 47 ± 2 °C. Aging started when the vials were placed at these temperatures (T_0). Three samples were analyzed at each time interval. The droplet size distributions of the freshly prepared emulsion were monomodal (Mastersizer 3600, Malvern) with volume-surface average droplet diameters (D_{32}) of 1.20 ± 0.01 and 0.36 ± 0.01 μm and spans of 1.77 and 1.32 for the low- and high-pressure emulsions, respectively. Low-pressure and high-pressure emulsions were designed as large droplet emulsions (LE) and small droplet emulsions (SE). The size distribution of the emulsions initially remained constant. Coalescence was detected in LE between the 16th and 21st days at 37 °C and between the 10th and 15th days at 47 °C. However, coalescence was limited and no phase separation was observed. SE were less stable, and coalescence began between the 11th and 16th days at 37 °C and was detected within 8 days at 47 °C. At that temperature phase separation occurred on the 10th and 15th days, and the emulsions were rehomogenized with the polytron homogenizer (30 s, 23000 rpm) before any sampling.

Lipid Oxidation Measurements. Hydroperoxides were determined in triplicate on each vial according to the xylenol orange method of Jiang et al. (*21*) adapted to edible oils by Nourooz-Zadeh et al. (*22*). This method focuses on the oxidation of Fe^{2+} to Fe^{3+} by hydroperoxides and the formation of a colored complex with xylenol orange, which absorbs light at 560 nm. The results were expressed as millimoles of cumene hydroperoxide equivalents per kilogram of oil (mmol of cumene HPX/kg) from a calibration curve of cumene hydroperoxides in 2-propanol.

Pentane development was followed by static headspace gas chromatography analysis. After 15 min of vial equilibration at 50 °C, 500 μL of headspace gas was injected with a gastight syringe into the inlet port equipped with an 800 μL splitless liner of a gas chromatograph (GC) (HP 5890 series II, Hewlett-Packard). The GC was fitted with a DB5 column (J&W Scientific, Folsom, CA), 30 m in length, 0.32 mm i.d., 1 μm film thickness, and a flame ionization detector at 250 °C. The initial and final oven temperatures were, respectively, 40 and 140 °C, with a rate of 5 °C min^{-1}. The flow of hydrogen carrier gas was 2.3 mL/min. Pentane was identified by comparison of its retention time with that of authentic reference pentane (1.8 min) and by GC-MS. The peak area was integrated (APEX software) and expressed in mV·s.

Front-Surface Fluorescence Spectroscopy. Front-surface fluorescence spectra were obtained with an SLM 4800C (Aminco) spectrofluorometer fitted with excitation (35°) and emission (vertical) polarizers and with a variable angle front-surface accessory set at 56°. The slit widths were set at 4 nm in both excitation and emission pathways. The samples were put in a quartz cell with a 1 mm optical pathway. The spectra were collected at 20 ± 1 °C in triplicate using three aliquots of each sample. Emission spectra of tryptophan were recorded from 300 to 400 nm (step, 1 nm; averaging, 2) with the excitation wavelength set at 290 nm. Emission spectra of later products of oxidation were recorded from 370 to 600 nm (step, 1 nm; averageing, 2) with the excitation wavelength set at 355 nm. All spectra were divided by the signal of rhodamine B in ethanol (2 g/L) in reference cell, and replicate spectra varied only slightly. Fourth derivatives of mean tryptophan spectra were calculated according to the Stavitzky−Golay modified procedure with a 16.2% smoothing (Peakfit software, 4th version, Jandel). The ratio of the intensities at 316 and 332 nm of the fourth-derivative spectra (d^4_{316}/d^4_{332}) was calculated by applying the peak−peak method (*23*).

Statistical Analysis. Two-way analysis of variance was performed according to the GLM procedure with least-squares means effects, using SAS system software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Hydroperoxides and Pentane Measurements. The storage of emulsions at 37 or 47 °C leads to lipid oxidation. Hydroperoxides soared to ∼120 mmol of cumene HPX/kg during the first day of aging, then increased steadily, and reached ∼180−200 mmol of cumene HPX/kg after several days of aging (Figure 1a). Effect of neither temperature nor droplet size was noticed on the time scale used ($p > 0.05$). In contrast to the primary products of oxidation, pentane rose linearly throughout the experiment with a 2-fold greater rate at 47 °C compared to that at 37 °C (Figure 1b). No dependence on the droplet size of the emulsions was observed ($p > 0.05$).

Fluorescent Pigments. The formation of fluorescent chromophores during aging was assayed using front-surface spectroscopy. The excitation wavelength was set at 355 nm as determined from preliminary experiments and in agreement with published data (*16*). Freshly prepared emulsions displayed only a broad and very low fluorescence emission (Figure 2). Upon aging, fluorescence emission rose progressively. The maximum emission wavelength was initially at 410 nm, but a shoulder at 425 nm became more prominent. In contrast, no such fluorescence developed during aging of a control BSA solution (data not shown). According to our results, the reaction of BSA with unsaturated aldehydes (*24*) or oxidizing linoleic acid (*13*, *25*) produced fluorescent products with excitation maxima at 350−380 nm and emission maxima at 420−450 nm. This fluorescence is attributed to the formation of lipid-oxidation-generated fluorescent compounds resulting from the reaction of hydroperoxides or non-radical secondary products of

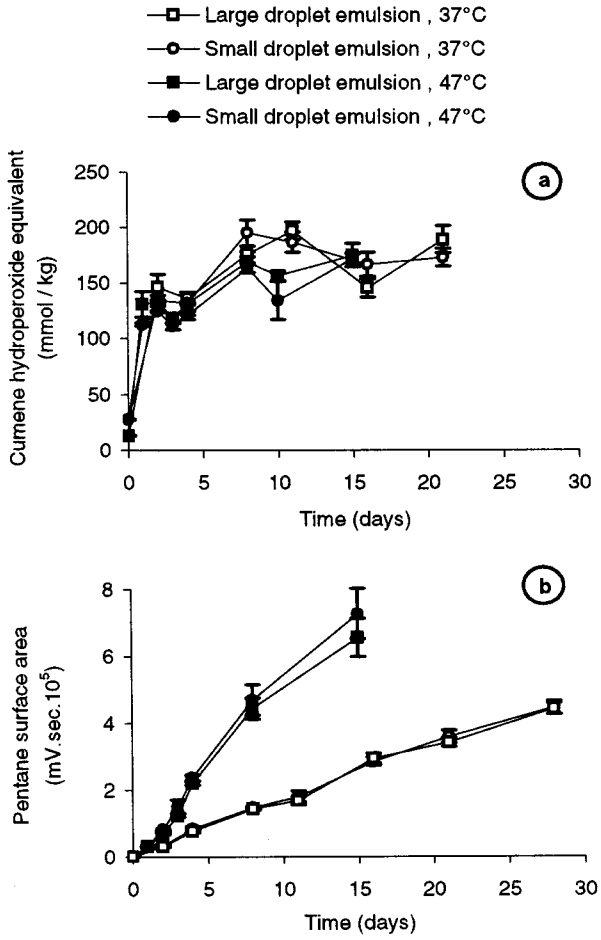

Figure 1. Hydroperoxides (a) and pentane (b) development during aging of emulsions ($n = 3$).

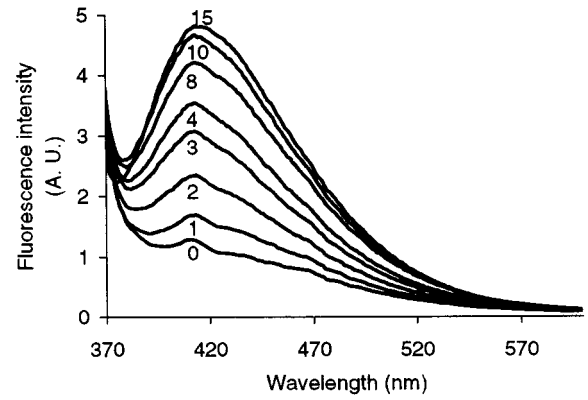

Figure 2. Front-surface emission fluorescence spectra of small droplet emulsions aged at 47 °C from 0 to 15 days. Excitation wavelength = 355 nm. Each spectrum is the average of three spectra.

lipid peroxidation with primary amines of proteins (*14*, *26*). In this way, a significant decrease in lysyl residues was measured in oxidizing emulsions (*3*). The wavelengths of emission and excitation maxima of lipid-oxidation-generated fluorescent compounds depend on the chemical species present in the reactive medium (*25*, *27*) and on the chromophore environment (*28*). They also increase with fatty acid unsaturation (*26*) and with reaction progress (*29*). Relatively short excitation and emission wavelengths were found in our experiments. Accordingly, linoleic acid, which contains only two double bonds, is the substrate of oxidation in sunflower

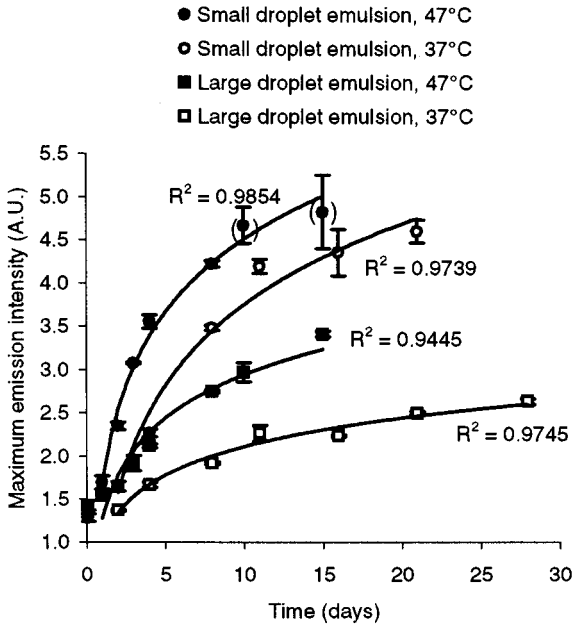

Figure 3. Time plots of maximum fluorescence emission during aging of emulsions. Excitation wavelength = 355 nm. Each point is the average of three repetitions. Points in parentheses indicate phase separation.

oil, and the earliest stage of the reaction of lipids with the protein was probably measured.

Fluorescence intensities at maximum emission wavelength, of the fluorescent compounds produced in oxidizing emulsions, were plotted as functions of time, droplet size of the emulsions, and temperature of storage (Figure 3). Best fits were obtained with logarithmic curves. The rate of fluorescence development varied with temperature ($p < 0.05$) in compliance with Arrhenius' law, as observed by Liang and Lin (*18*), and in agreement with the pentane measurements. It also varied with the droplet size of the emulsions: it was higher in SE than in LE ($p < 0.05$). When oxidized LE was rehomogenized to obtain SE, constant fluorescence intensity was recorded. This demonstrates that more fluorescent pigments are produced in SE due to the greater interface area, which favors interactions and reactions between oxidizing lipids and protein.

Tryptophan Fluorescence in Freshly Prepared Samples. The fluorescence intensity of the tryptophanyl residues in BSA decreased in the following order: solution > LE > SE (Figure 4a). It was lower in emulsions than in BSA solution, most likely because the oil droplets induce diffusion and diffraction of the excitation and emission radiation leading to inner-filter effects. Diffusion and diffraction phenomena vary with droplet size and wavelength. At 290 nm, the excitation wavelength of Trp residues, the turbidity of 0.5 μm emulsions is higher than the turbidity of 2 μm emulsions (*30*). This could explain, in part, the higher fluorescence intensity of tryptophan in LE than in SE, in our experiments. The wavelength at maximum fluorescence emission (λ_{max}) was blue-shifted (4 nm) for SE, compared to LE and BSA solution (Figure 4a). This shift is assigned to a displacement of protein Trp to a more hydrophobic location when the protein adsorbs at the interface, stabilizing the emulsion (*31*). Castelain and Genot (*19*, *20*) already noticed a blue shift upon the adsorption of BSA in dodecane-in-water emulsions. The shift is directly proportional to the ratio of adsorbed/total BSA ratio. In LE the shift is not noticeable because

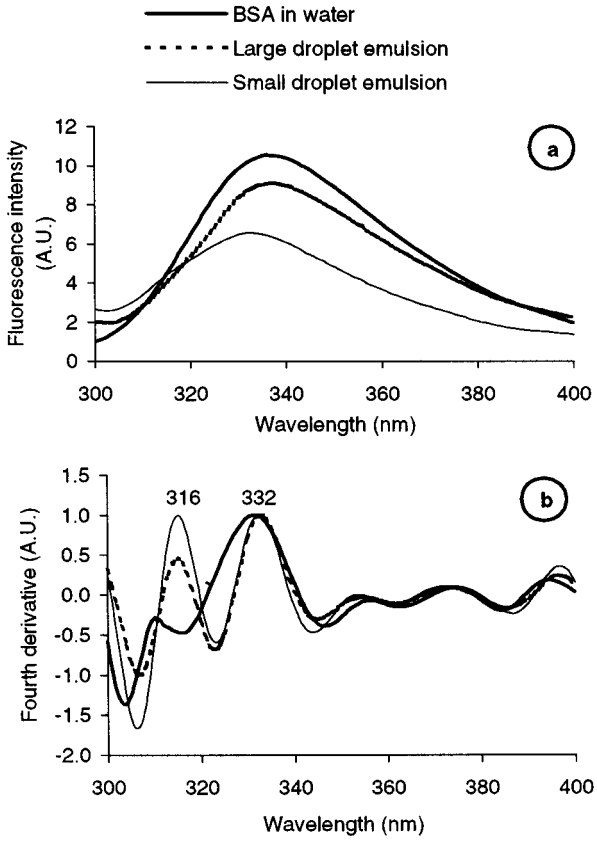

Figure 4. Emission (a) and fourth-derivative emission (b) fluorescence spectra of freshly prepared BSA in solution and in emulsions. Excitation wavelength = 290 nm. Each spectrum is the average of three spectra.

the interface area is too small (~5 m^2/cm^3 of oil) compared to the total protein amount available, so a small fraction of BSA is adsorbed at the interface and the Trp residues present in nonadsorbed BSA make a major contribution to the total fluorescence. In SE, the interface area is larger (17 m^2/cm^3 of oil), the contribution of Trp in the hydrophobic environment due to BSA adsorption is enhanced, and the shift is noticeable.

However, a single and broad peak characterizes the basic emission spectrum, which does not allow the distinguishing of the Trp in hydrophilic and hydrophobic environments. Consequently, to deconvolute the contribution of Trp in the two environments, differentiation of the basic spectra was done (*23*). As second derivatives of Trp emission spectra gave only small shoulders to the basic signal, the fourth derivatives were calculated. The fourth-derivative spectra clearly differentiate emulsions from BSA solution (Figure 4b). The peak at 332 nm, found both in BSA solution and in emulsions, is assigned to Trp residues of BSA in an aqueous environment. This peak is broader for BSA in solution, most likely because the protein in water contains two Trp residues with similar but not exactly equivalent environments (*32*, *33*). A second peak at 316 nm was found solely in emulsions. This peak is attributed to Trp residues in a more hydrophobic environment and supports the assumption of modifications of BSA conformation upon its adsorption onto the oil−water interface. It is more intense in SE than in LE because in SE the contribution to total fluorescence of Trp residues in hydrophobic environment is enhanced. To summarize, in BSA-stabilized emulsions, Trp residues may occur in three environments: (i) Trp in the roughly hydrophilic

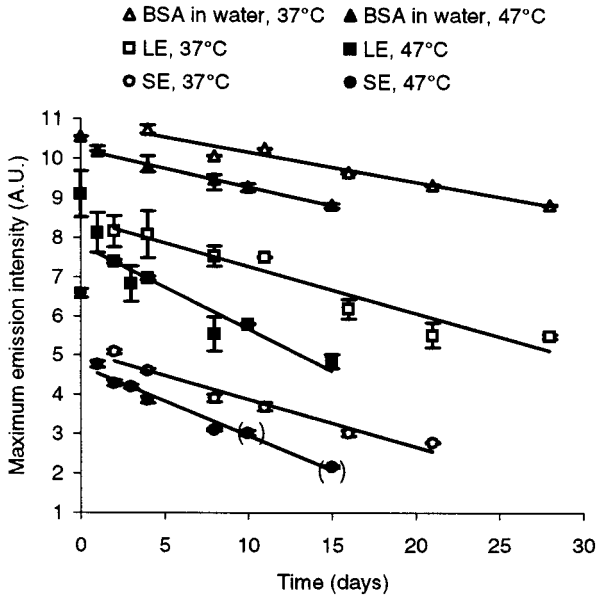

Figure 5. Maximum fluorescence emission of tryptophanyl residues of BSA during aging of samples (SE, small droplet size emulsion; LE, large droplet size emulsion). Excitation wavelength = 290 nm. Each point results from the average of three repetitions. Points in parentheses indicate phase separation.

environment contained in nonadsorbed BSA, (ii) Trp in a hydrophilic environment from adsorbed BSA, and (iii) Trp in a hydrophobic environment, either buried in the protein structure or in contact with the lipid phase, from adsorbed BSA. The first two Trp classes exhibit fourth-derivative spectra with a maximum located at 332 nm; the last class is characterized by the maximum at 316 nm. The relative intensities of these maxima (d^4_{316}/d^4_{332}) will provide information about Trp partition in hydrophobic (316 nm) and aqueous (332 nm) regions as shown in the next paragraph.

Tryptophan Fluorescence in Aging Samples. As for fluorescent pigments, maximum fluorescence emission (I_{max}) of Trp residues was plotted versus aging time (Figure 5). I_{max} decreased with time in BSA solution and in LE and SE. The decrease was linear ($r^2 > 0.95$) in BSA solution and similar at 47 and 37 °C ($p > 0.05$), with slopes of −0.09 and −0.08, respectively. The relative absence of lipids in BSA solutions greatly reduces the possibility of tryptophan oxidation phenomena, which is confirmed by lack of O_2 consumption (data not shown). The decrease can be explained by reactions such as aggregation and/or polymerization of the protein (*2*, *3*), which could lower the Trp fluorescence quantum yield. An increased inner-filter effect could also explain the decrease in fluorescence because the turbidity of the protein solution slightly increased during aging (data not shown).

In the emulsions, I_{max} dropped during the first day of aging. The drop was more pronounced for SE than for LE. Tryptophan oxidation is probably involved, induced by the free radicals produced during the first stage of lipid oxidation (*34*). Accordingly, a sharp increase in hydroperoxide content was simultaneously observed (Figure 1a). Davies et al. (*10*, *11*) also showed a fall in Trp fluorescence, assigned to Trp oxidation, upon exposure of BSA to oxygen radicals. After this drop, a slight and linear decrease in Trp fluorescence was observed with slopes greater than those calculated for BSA solution ($p < 0.5$). At 47 °C the slopes, −0.21 (r^2 =

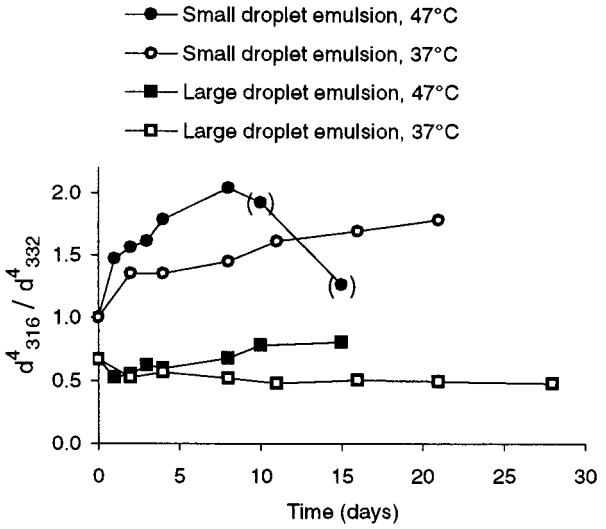

Figure 6. Ratio of fourth-derivative spectra at 316 and 332 nm during aging of emulsions. Excitation wavelength = 290 nm. Each point is the average of three repetitions. Points in parentheses indicate phase separation.

0.90) for LE and −0.18 ($r^2 = 0.97$) for SE, were greater than those at 37 °C, −0.12 ($r^2 = 0.93$ and 0.96 for LE and SE, respectively) ($p < 0.05$). Thus, in addition to protein aggregation and polymerization phenomena observed for BSA solution, oxidation of Trp residues probably continues in emulsions and is enhanced when the temperature is higher.

The fourth derivatives of Trp emission spectra were calculated for all experimental conditions. The derivative spectra of BSA solutions (data not shown) did not change whatever the temperature and time of aging: no major changes in Trp environment occurred. As observed in freshly prepared emulsions, the derivative spectra of aged emulsions presented two peaks at 316 and 332 nm. The ratio, d^4_{316}/d^4_{332}, stayed lower in LE than in SE (Figure 6). In SE, d^4_{316}/d^4_{332} increased upon aging and then plunged after 10 days at 47 °C, probably related to phase separation. The increase, more marked at 47 °C than at 37 °C, is attributed to an increase in the hydrophobic to hydrophilic Trp ratio during aging. Different hypotheses can be argued: (i) The quantity of BSA that adsorbs at the oil−water interface rises, so more Trp residues are exposed to a hydrophobic environment. (ii) The protein aggregates or polymerizes, leading to quenching of Trp in the hydrophilic environment. (iii) Trp residues in the hydrophilic environment are submitted to oxidative degradation. In this experiment, which was carried at a too long time-scale (several weeks) for adsorption phenomena to remain significant, a decrease of Trp fluorescence intensity was observed (Figure 5) which is attributed to degradation of the amino acid residues during aging (see above). Consequently, only the second and third hypotheses could explain both fluorescence intensity decrease and increase of d^4_{316}/d^4_{332} in SE. In LE, the amount of protein in solution is high, and the BSA in solution makes a major contribution to the total fluorescence. In these emulsions, d^4_{316}/d^4_{332} remained rather steady during aging (Figure 6). This stability indicates that the structure of nonadsorbed BSA did not change significantly during aging, despite likely aggregation and/or polymerization inducing a decrease in total fluorescence. We can thus assume that the global modifications of the Trp environment in SE evidenced by fourth-derivative spectra involved mainly oxidative degradation of the residues in the hydrophilic environment of the BSA molecules adsorbed at the oil−water interface. These modifications can be paralleled to the progressive appearance of fluorescent pigments, which is favored in SE.

Conclusion. Front-surface fluorescence spectroscopy is an efficient technique to evaluate emulsion modifications upon aging. The formation of lipid−protein oxidation products, aggregation of BSA in the aqueous phase, and structural and/or oxidative modifications of the protein at an oil−water interface have been studied simultaneously. Using this noninvasive method, further investigations are now being carried out to more accurately characterize the earliest stage of emulsion aging. Moreover, further research is needed to clarify the modifications that have affected the adsorbed and nonadsorbed proteins upon aging.

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