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Oxidative Stability of Bovine Serum Albumin- and Sodium Caseinate-Stabilized Emulsions Depends on Metal Availability

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Proteins often stabilize food emulsions and are also able to promote or delay lipid oxidation in complex systems. The purpose of this work was to investigate the relationship between metal ion availability and oxidative stability of oil-in-water emulsions stabilized by bovine serum albumin (BSA) or sodium caseinate (NaCas). Emulsions with similar and stable droplet size distributions were prepared with stripped sunflower oil (30 vol %) and protein solutions (20 g L^{-1}; pH = 6.5). In the absence of the water-soluble metal chelator EDTA, oxygen uptake, conjugated dienes, and volatile compounds developed faster in NaCas-stabilized emulsions than in those prepared with BSA. This effect is attributed to the chelating properties of NaCas and to electrostatic interactions that attract some metal ions at the interface where they could initiate lipid oxidation. When EDTA (100 μM) was present, oxidation was delayed to a greater extent in emulsions made with NaCas than in BSA stabilized emulsions. These conditions probably enabled NaCas to exert free-radical-scavenging activity.

KEYWORDS: Lipid oxidation; oil-in-water emulsions; milk proteins; interface; iron

INTRODUCTION

Lipid oxidation is a major cause of food quality deterioration. It leads to the development of off-flavors (rancidity) and potentially toxic compounds. The main rules of lipid oxidation in bulk oils are known and recent studies have focused on lipid oxidation in oil-in-water (o/w) emulsions (*1*). In many foods, the lipid phase is dispersed as oil droplets in an aqueous matrix. Oxidation phenomena in emulsions mimic what happens in food products. Lipid oxidation is favored because the droplets present a large contact surface between oxidizable fatty material and the water-soluble chemical compounds, namely, oxygen and ions of transition metals, which participate in the initiation and propagation of oxidation reactions (*2*, *3*).

Proteins are generally regarded as safe (GRAS) food ingredients and they are widely used as emulsifiers in food products (*4*). Many proteins also exert antioxidant activity when added in the aqueous phase of o/w emulsions stabilized by small surfactants such as Tweens or phospholipids. The antioxidant activity of milk proteins was demonstrated in various studies (*5*−*9*). In particular, caseins and casein-derived phosphopeptides inhibited formation of lipid hydroperoxides and hexanal in Brij-stabilized corn oil emulsions (*6*). The ability of casein phosphorylated groups to chelate pro-oxidant metals ions may explain this inhibition, but free-radical-scavenging activity could also be involved (*6*, *7*). The high molecular weight (HMW) fraction of whey proteins lowered the formation of lipid peroxides and thiobarbituric acid reactive substances (TBARS) when added to salmon oil emulsions stabilized by Tween 20 (*9*). This antioxidant activity was concentration-dependent and was attributed to several mechanisms including hydrogen-donating capacity of protein thiol (SH) groups, free-radical-scavenging activity of other amino acid residues, and ability to retain soluble iron far from the oil droplet surface. Reactivity of protein SH groups was also involved in the antioxidant activity of bovine serum albumin (BSA) and other proteins (*10*).

Some studies focused on the oxidative stability of emulsions in which proteins were used as emulsifiers (*11*−*13*). In these studies, lipid oxidation was assumed to be mainly governed by electrostatic interactions between metallic ions and the emulsion droplet interfacial membrane. For instance, lipid oxidation in whey protein isolate (WPI) stabilized emulsions was much lower at pH values below the protein's isoelectric point (pI), probably because the emulsion droplets were positively charged and repelled cationic iron (*11*, *12*). However, oxidation rates in WPI-, sweet whey-, β-lactoglobulin-, or α-lactalbumin-stabilized emulsions did not parallel their surface charge (*12*). Similar results were found with casein-, soy protein isolate-, and WPI-stabilized emulsions (*13*). Thus, the charge of emulsion droplets is not the only factor influencing the oxidative stability of lipids in protein-stabilized emulsions. In addition to protein ability to chelate metal ions, scavenge free radicals, or react with primary and secondary products of oxidation, other factors such as the physical characteristics of the oil−water interface, thickness, permeability to small molecules, and viscosity can also modulate lipid oxidation (*6*, *14*, *15*).

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In emulsified systems and in other model systems, some proteins generally considered as antioxidant were pro-oxidant under specific conditions. For instance, when added to the aqueous phase of menhaden oil emulsions stabilized by Tween 20, WPI favored formation of lipid peroxides and TBARS. However, the reason for this enhanced oxidation was unclear (*11*). Other examples showed that proteins can behave as anti- or pro-oxidant, depending on physicochemical conditions of the systems (*16*−*24*).

BSA is a globular structured protein of high molecular mass, with antioxidant activity partly based on the reactivity of its SH group with free radicals (*10*). When it stabilizes o/w emulsions, it adsorbs at the surface of the oil droplets and changes its conformation, exposing hydrophobic amino acids, such as tryptophanyle residues, to a more hydrophobic environment (*25*, *26*). Sodium caseinate (NaCas) is a mixture of disordered proteins of relatively low molecular weights. They can prevent lipid oxidation, essentially by chelating metals ions, but other mechanisms such as free-radical scavenging may also be involved. As their hydrophobic and hydrophilic residues are clustered into large and separate domains, caseins adsorb to the oil−water interfaces through their hydrophobic domain, whereas the hydrophilic domain forms a tail protruding in the aqueous phase (*27*).

The aim of this paper is to demonstrate that the oxidative stability of o/w emulsions stabilized by proteins such as BSA or NaCas is related to both availability of metal ions and protein properties. Oxidative stability was followed by different methods: oxygen (O_2) uptake gave an overall view of oxidation development in the emulsions; conjugated dienes (CD) and volatile compounds (pentane, hexanal, 1-octen-3-ol, and 2-(*E*)-octenal) are markers of genesis of primary and secondary oxidation products, respectively. The influence of the water soluble chelator, ethylenediamine-tetraacetic acid (EDTA), on the oxidative stability of emulsions was evaluated with O_2 uptake measurements. Chelating properties of the proteins and emulsion droplet charges were also measured.

MATERIALS AND METHODS

Materials. Commercial sunflower oil was stripped of tocopherols, mono- and diacylglycerides, and free fatty acids using adsorption chromatography on an alumina column (Alumina N, Akt. I, ref 020287; ICN Biomedicals Inc., Aurora, OH). Pentane used as eluting solvent was evaporated from stripped oil under vacuum and flushed with nitrogen until no trace could be detected by SPME-GC analysis. Stripped oil contained 8.5 mg of residual tocopherols per kilogram of oil. Its fatty acid composition was 63.1% 18:2 n-6, 24.6% 18:1 n-9, 6.7% 16:0, 4.9% 18:0, 0.3% 20:0 (percentage of total peak area, determined by gas chromatography of methyl esters). Other fatty acids represented less than 0.4%. Powdered BSA (ref 103703; 95−98% purity) was obtained from ICN Biochemicals Inc. and NaCas (92.7% purity; 46% α_S-casein, 40% β-casein, 14% κ-casein) from Armor Protéines (St-Brice-en-Coglès, France). NaCas and BSA powders contained 96 ± 2 μmol kg^{-1} and 163 ± 1 μmol kg^{-1} iron, respectively, as determined by atomic absorption spectroscopy. Sodium azide (NaN_3, purity ≥99%) and EDTA were purchased from Merck (Strasbourg, France). Hexanal (98%), 1-octen-3-ol (98%), 2-(*E*)-octenal (94%), 1,10-phenantroline monohydrate (99%), hydroquinone (99%), and ammonium iron (II) sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2$, $6H_2O$] (>99%) were purchased from Aldrich (St Quentin Fallavier, France). Pentane and 2-propanol (analytical grade) were purchased from Carlo Erba (Val de Reuil, France). Ultrapure water (18 MΩ) prepared with Milli-Q system (Millipore, Billerica, MA) was used throughout.

Methods. *Preparation, Characterization, and Storage of Emulsions.* The day before emulsion preparation, BSA or NaCas solutions (20 g L^{-1}; 0.4 g L^{-1} NaN_3; pH adjusted to 6.5) were prepared and gently stirred overnight at +4 °C to entirely dissolve the proteins without foam formation. The solutions were equilibrated at room temperature just before use and their pH was adjusted to 6.5 by addition of either NaOH or HCl (1 mol L^{-1}). O/W emulsions were prepared with 30 vol % stripped sunflower oil and 70 vol % protein solutions. When added, EDTA was dissolved in the protein solution to obtain a final concentration of 100 μM in the emulsions. The two phases were premixed for 2 min at 24 000 rpm using a rotor-stator homogenizer fitted with a 12-mm diameter head (Polytron PT 3000, Kinematica, Littau, Switzerland). The coarse emulsions were then homogenized for 4 min at 35 bar through a one-stage low-pressure valve homogenizer (A0812W-A-CD, Stansted Fluid Power, Stansted, U.K.).

The size distribution of the oil droplets in the emulsions was measured immediately after homogenization with a Mastersizer 3600 laser light scattering instrument (Malvern Instruments, Worchester, U.K.). It was daily checked to monitor emulsion stability and reported as volume−surface mean diameter ([d3,2]; μm) and span ([d(v,0.9) − d(v,0.1)]/d(v,0.5)). The charge of the emulsion droplets (zeta potential, ξ; mV) was also measured after homogenization, with a Z 3310 Zetaphoremeter III (CAD instrumentation, Les Essarts Le Roy, France).

Aliquots (1.5 mL) of emulsions were distributed in 22.4-mL headspace vials sealed with Teflon/silicon septa and aluminum crimp caps. The vials were rotated in the dark at 50 ± 2 °C, at 20 rpm, with a test tube rotator (Labinco B. V., Ac Breda, The Netherlands) oriented at 30 degrees versus vertical position. Aging started (t_0) when the vials were put in the thermostated chamber. pH of the samples was measured with a Metrohm-691 pH-meter fitted with an electrode suitable for measurements in emulsions (Metrohm 6.0258.000, Herisau, Switzerland).

Measurement of Lipid Oxidation. O_2 concentration in the headspace of the samples was measured with a HP5890-serieII gas chromatograph (Hewlett-Packard, Böblingen, Germany) paired with a thermal conductivity detector (TCD). A sample (100 μL) of the headspace was injected with a gastight syringe into the injector (split rate: 30 mL min^{-1}, injector temperature: 50 °C) connected to a fused silica PLOT column (molsieve 5 Å, 10-m length, 0.32-mm i.d., 0.30-μm film thickness; Varian, Les Ulis, France). Analysis was performed in isothermal mode at 50 °C with TCD temperature set at 125 °C. The flow rate of helium carrier gas was 2 mL min^{-1}. Peaks were integrated with Borwin software (JMBS développements, Fontaine, France). The O_2 peak area was compared to the area obtained for the same volume of surrounding air. The total amount of oxygen in the vials was then calculated from the theoretical concentration of O_2 in ambient air and the headspace volume and subtracted from the initial amount of O_2 in the headspace of the vials. The results were expressed in millimoles of consumed O_2 per kilogram of oil (mmol O_2 kg^{-1}oil).

To evaluate the formation of primary products of lipid oxidation in the emulsions, conjugated dienes (CD) were evaluated according to the method described by Lethuaut et al. (*3*). Aliquots of emulsions were diluted in 2-propanol to obtain lipid concentrations of 250 mg L^{-1}. The solutions were centrifuged for 4 min at 5600 × g. The absorbance of the supernatants were measured at 233 nm with a UV−vis double beam spectrophotometer (Perkin-Elmer Lambda 12, Norwalk, CT). Reference cell contained water in 2-propanol in the same proportions as in samples. Results were expressed in millimoles of conjugated dienes per kilogram of oil (mmol CD kg^{-1}oil) using 27 000 M^{-1} cm^{-1} as the molar extinction coefficient of CD at 233 nm (*28*).

Four volatile compounds, namely, pentane, hexanal, 1-octen-3-ol, and 2-(*E*)-octenal, were selected as markers of secondary products of lipid oxidation. They were analyzed by gas chromatography (GC) of the volatile compounds sampled in the headspace of the vials equilibrated at 50 °C, with a solid-phase microextraction (SPME) fiber. The fiber, coated with polydymethylsiloxane (PDMS) (10-mm long, 100-μm film thickness, Supelco, Bellefonte, PA), was exposed in the headspace for 5 min at 50 ± 2 °C. As 15 min were required to equilibrate at 50 °C the vials that were initially at ambient temperature, measurements made on the freshly prepared emulsions corresponded to 15 min aging. The fiber was then transferred to the injection port of the GC (HP5890-serie II) equipped with a 0.75-mm i.d. liner and set at 260 °C. The purge was kept off during the first 5 min of desorption to ensure complete injection of the volatiles in the column (hydrogen

split flow: 30 mL min^{-1}). Volatiles were separated on a DB 624 fused silica capillary column (30-m length, 0.32-mm internal diameter, 1.8-μm film thickness; J&W Scientific, Chromoptic, Auxerre, France). Hydrogen at 2 mL min^{-1} was used as the carrier gas and the temperature program was as follows: 5 min at 35 °C, 10 °C min^{-1} until 220 °C, and 4 min isothermal. The eluted compounds were detected with a flame ionization detector (FID) set at 250 °C and hydrogen and air flows set at 23 and 230 mL min^{-1}, respectively. The volatile compounds were identified (i) by comparison of their retention times with those of authentic reference standards and (ii) by combined GC (HP5890-serie II) mass spectrometry (HP5971A) performed in the conditions similar to the GC-FID with the exception that helium at 1 mL min^{-1} was used as the carrier gas. Mass spectra were obtained with a 70 eV electron impact ionization and continuous scanning from *m*/*z* 33 to 250 at a scan speed of 0.4 scan s^{-1}. They were compared to spectra found in available libraries (NBS, NIST, TNO). Peak area values obtained with the FID detector were integrated with Borwin software and compared with those obtained by direct injection in the GC in splitless mode of known amounts (0.4−40 ng) of pentane, hexanal, 1-octen-3-ol, and 2-(*E*)-octenal in cyclohexane (calibration curves). Results were thus expressed in ng of volatile compound desorbed from the fiber.

The fibers used throughout this study were daily controlled by check of peak areas obtained after 5 min fiber exposure in the headspace of a test solution containing known amounts of the four volatile compounds and equilibrated at 50 °C.

Iron-Binding Properties of the Proteins. To evaluate the ability of the proteins to bind soluble iron, mixtures of the protein solution and known amounts of ferrous iron were filtrated through cutoff filters and the unbound metal was quantified (*29*). Ferrous iron was added to NaCas and BSA solutions (1 g L^{-1}, pH adjusted to 6.5) at concentrations ranging from 0.6 to 5.2 mg L^{-1}. After one night at room temperature, protein solutions were filtered through disposable cutoff filters (Vivaspin 2 mL; molecular mass cutoff: 10 kDa, Hannover, Germany) to remove proteins and protein-bound iron. The filtrate contains only the free iron. Free Fe^{2+} reacts with phenantroline to give a colored complex that is quantified by measurement of solution absorbance at 508 nm. One volume of phenantroline reagent (2.5 mM phenantroline, 18.2 mM hydroquinone in ethanol/water; 50/50; v/v) was added to one volume of the filtrate. The solution absorbance was read immediately at 508 nm against a blank. The concentration of free iron was determined from the calibration curve built with ammonium iron (II) sulfate hexahydrate solutions (1−10 mg L^{-1}). It was then subtracted from the concentration of added iron to obtain the concentration of bound iron. The experiment was also performed with pure water as a control.

Experimental Design and Data Treatment. Three BSA- and NaCas-stabilized emulsions were prepared separately and distributed in vials. At each aging time, one vial per experimental series was taken from the temperature-controlled chamber for analysis. Particle size distribution, pH, and volatile compound amounts were measured once on these vials. O_2 uptake and CD values were calculated from the average of, respectively, two and three measurements performed on each vial. Mean values (±standard deviations; $n = 3$) were then calculated from the values obtained on the triplicate emulsions. In the presence of EDTA, two emulsions were prepared with each protein. Thus, results of O_2 uptake are shown for each replicate. They represent averages of three measurements per vial. Concentrations of iron bound on the proteins are results of duplicate measurements performed on two separate preparations of protein solutions.

One-way variance analysis (ANOVA) was performed for each protein type to determine the effect of time of storage on O_2 uptake, conjugated dienes, volatile compounds, and pH. If significant effects were found ($p < 0.05$), least significance difference (LSD) tests were performed to discriminate means. When a statistically significant difference among the standard deviations at the 95.0% confidence level was found (variance check), the Kruskal-Wallis test was performed to confirm results obtained with standard statistical tests. For a given time of storage, mean values obtained on NaCas- and BSA-stabilized emulsions were compared with hypothesis tests. Analysis were performed with Statgraphics Plus 3.0 software (Manugistic, Rockville, MD) and the usual significance level was $p < 0.05$ unless otherwise stated.

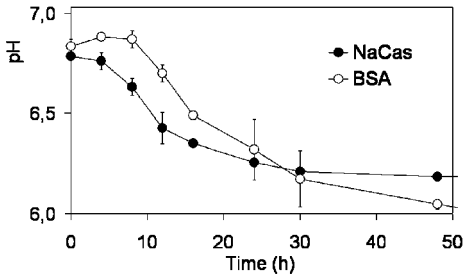

Figure 1. Evolution of pH during aging at 50 °C in the dark of 30% sunflower oil emulsions stabilized by bovine serum albumin (○) or sodium caseinate (●). Data points represent mean ($n = 3$) ± standard deviation.

RESULTS

Physicochemical Characterization of the Emulsions. As expected, the freshly prepared emulsions had very similar and monomodal droplet size distributions. For BSA-stabilized emulsions, the mean surface diameter [$d_{3,2}$] was 0.87 ± 0.04 μm and the span was 1.51 ± 0.06. For NaCas-stabilized emulsions, [$d_{3,2}$] was 0.92 ± 0.02 μm and the span was 1.43 ± 0.04. The droplet size distributions of the emulsions largely overlap each other and oil−water interface areas ($6/d_{3,2}$]) are very close. Consequently, differences in oxidative stability of the NaCas- or BSA-stabilized emulsions could not be attributed to differences in droplet size and interfacial surface area (*3*). The droplet size distributions of the emulsions remained constant even after 10 days of storage, and no phase separation nor creaming was observed, showing that the emulsions were physically stable and not destabilized during the rotating agitation at 50 °C.

NaCas-stabilized oil droplets were more negatively charged (−81.2 ± 5.1 mV) than the droplets stabilized by BSA (−21.7 ± 1.8 mV), as revealed by ξ-potential measurements.

The initial pH of the emulsions was slightly above the pH of the protein solutions (6.5): it was 6.75 ± 0.01 for NaCas- and 6.81 ± 0.04 for BSA-stabilized emulsions. During aging, the pH of the NaCas- or BSA-stabilized emulsions remained stable (homogeneous groups as revealed by LSD test) for the first 4 or 8 h, respectively, then decreased rapidly to finally decrease slowly after 30 h aging (**Figure 1**). pH values of 6.16 ± 0.03 and 6.02 ± 0.03 were reached after 48 h for the emulsions prepared with NaCas and BSA, respectively, but the pH continued to decrease slowly in both emulsions after 48 h (not shown).

Oxidative Stability of Emulsions Made with NaCas or BSA. O_2 uptake showed similar sigmoid kinetics in both emulsions (**Figure 2**). It increased slowly during the first 8 h of aging, then increased sharply to a plateau after 30 h. At this stage, O_2 uptake represented about 80% (around 340 mmol O_2 kg^{-1} oil) of the amount initially present in the headspace of the emulsions, irrespective of the stabilizing protein. This value remained fairly constant until the end of the experiment. A significant effect of the type of emulsifying protein on the amount of consumed O_2 was pointed out. From 4 h aging of the emulsions, O_2 uptake was significantly lower ($p = 0.0026$) in the BSA-stabilized emulsions (16.4 ± 0.7 mmol O_2 kg^{-1}oil) compared to NaCas (40.8 ± 0.2 mmol O_2 kg^{-1}oil). The difference remained highly significant ($p < 0.01$) until 48 h aging. Half the maximum of O_2 uptake was reached after 15 h in the BSA-stabilized emulsions whereas only 11 h was needed to reach this half level with NaCas. Until the plateau was

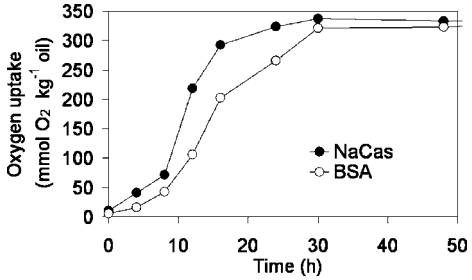

Figure 2. Time course of O_2 uptake during aging at 50 °C in the dark of 30% sunflower oil emulsions stabilized by bovine serum albumin (○) or sodium caseinate (●). Data points represent mean ($n = 3$) ± standard deviation, error bars are within symbol.

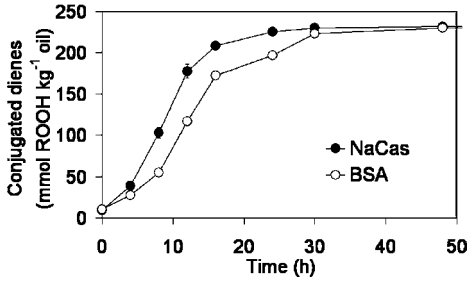

Figure 3. Formation of conjugated dienes during aging of 30% sunflower oil emulsions stabilized by bovine serum albumin (○) or sodium caseinate (●). Data points represent mean ($n = 3$) ± standard deviation, some error bars are within symbol.

reached, O_2 uptake remained significantly higher in the emulsions prepared with NaCas than in those prepared with BSA.

Formation of CD followed very similar trends (**Figure 3**). CD increased slowly first and then soared to finally level off progressively to a plateau when around 230 mmol CD kg^{-1}oil was produced, irrespective of the emulsifying protein. This level remained constant even for a long time of storage (10 days) as previously observed with results from O_2 uptake. Results were highly reproducible and demonstrate, that until 48 h of storage, significantly ($p < 0.05$) lower amounts of primary products of oxidation (CD) were produced in the emulsions made with BSA than in those made with NaCas. Twelve hours of aging was required to reach half the maximum level of CD in the case of BSA as compared with only 9 h in the case of NaCas.

Twenty volatile compounds were identified as secondary products of oxidation in the headspace of the oxidizing emulsions (data not shown). Pentane, hexanal, 1-octen-3-ol, and 2-(*E*)-octenal were chosen as markers of lipid oxidation. Pentane and hexanal were the major compounds produced and were often used as indexes of lipid oxidation in oils or fatty materials containing high level of linoleic acid (*3*, *6*, *13*, *30*−*32*). 1-Octen-3-ol, 2-(*E*)-octenal, and hexanal are involved, among others, in the development of off-flavors during lipid oxidation (*14*, *33*). These four volatile compounds were even detected (from 0.1 to 10 ng) in the headspace of the fresh emulsions equilibrated 15 min at 50 °C before SPME-GC measurements. Quantities of each volatile compound detected in the headspace of the emulsions were plotted versus time (**Figure 4**). The shapes of the curves differed for each volatile compound, highlighting the complexity of lipid oxidation kinetics and production of volatile secondary products of oxidation. The amount of hexanal (**Figure 4-top**) desorbed from the SPME fiber increased rapidly during the first 24 h and then continued to increase slowly even after 10 days (data not shown). Pentane quantity increased linearly as a function of time throughout incubation. 2-(*E*)-Octenal amount remained very low during the first 16 h and

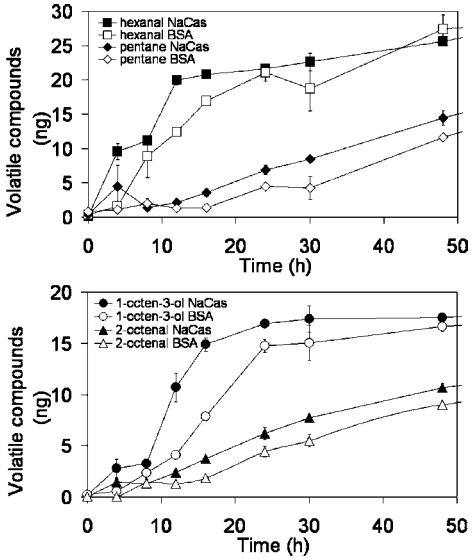

Figure 4. Evolution of pentane and hexanal, (top), 1-octen-3-ol and 2-(*E*)-octenal (bottom) in the headspace of 30% sunflower oil emulsions stabilized by BSA or NaCas, during aging at 50 °C, in the dark ($n = 3$). Data points represent mean ($n = 3$) ± standard deviation.

then increased progressively during aging (**Figure 4-bottom**). The amount of 1-octen-3-ol increased slowly during the first 8 h, then increased sharply until 24 h, and finally remained constant. In agreement with measurements of CD and O_2, during the first 24 h of aging, the quantities of hexanal were significantly different in the headspace of the emulsions prepared with NaCas or BSA. For instance, after 4 h aging at 50 °C, the amount of hexanal desorbed from the SPME fiber was 1.6 ± 0.1 ng for BSA-stabilized emulsions compared to 9.6 ± 1.4 ng for NaCas-stabilized emulsions ($p < 0.01$). After 12 h aging, the amount of detected hexanal was 12.4 ± 0.3 ng with BSA and 20.0 ± 0.7 with NaCas ($p < 0.005$). After 30 h, hexanal amounts in both emulsions were no longer significantly different ($p = 0.256$): they attained 18.7 ± 3.2 ng and 22.6 ± 1.3 ng with BSA and NaCas, respectively. Thereafter, with longer incubation time, the amount of hexanal detected in the headspace remained similar irrespective of the emulsifying protein. Concerning the three other volatiles, differences between NaCas- and BSA-stabilized emulsions were often found nonsignificant during the first 8 h of the kinetics, because of the small amounts of volatiles detected and large variation coefficients. They became significant ($p < 0.05$) between 12 and 48 h aging. When the emulsions were stored for longer periods (>48 h, data not shown), the amounts of volatile compounds were similar whatever the emulsifying protein.

As expected, in the presence of 100 μM EDTA the rise of O_2 uptake was delayed by several hours in both types of protein-stabilized emulsions (**Figure 5**). In the BSA-stabilized emulsions, the rise of O_2 uptake occurred after about 16 h aging at 50 °C. It reached half its maximum level after 35 h and leveled off after 56 h incubation. In emulsions made with NaCas, O_2 uptake started to increase after 52 h, reached half its maximum level after 80 h, and leveled off after 100 h incubation.

Iron-Binding Properties of the Proteins. When 0.7−5.2 mg L^{-1} iron was added to the 1 g L^{-1} NaCas solution, nearly all the metal was bound by the proteins (**Table 1**). The difference between bound iron and total added iron accounted for experimental losses as shown by values obtained on control. This result is in accordance with previous studies that demonstrated the efficient iron-chelating properties of caseins (*34*−*35*). Indeed, the total iron-binding capacity of caseins was not

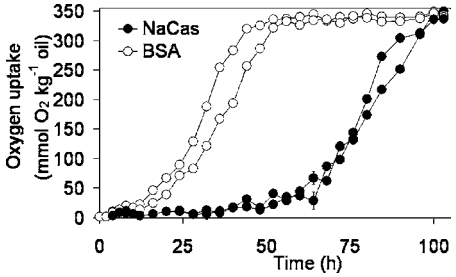

Figure 5. Time course of oxygen uptake during aging at 50 °C in the dark of 30% sunflower oil emulsions stabilized by bovine serum albumin (○) or sodium caseinate (●), in the presence of EDTA 100 μM. For each type of protein-stabilized emulsions, results are presented for the duplicate samples.

Table 1. Retention of Ferrous Iron by Solutions of BSA or NaCas

	bound iron (mgL^{-1})		
total iron (mgL^{-1})	control	BSA (1 g L^{-1})	NaCas (1 g L^{-1})
0.68	0.01 ± 0.02	0.60 ± 0.01	0.63 ± 0.02
1.34	0.02 ± 0.00	0.97 ± 0.01	1.29 ± 0.02
2.65	0.13 ± 0.09	1.23 ± 0.07	2.60 ± 0.01
5.17	0.07 ± 0.04	1.38 ± 0.03	5.1 ± 0.03

reached in the present study, but the results show that all the soluble iron that could be present in the emulsions was likely bound by caseins. When soluble iron was added to BSA solution, bound iron increased progressively with total iron concentration. A maximum of 1.38 mg of iron was retained per gram of protein, that is, around 1.7 mol iron per mol of protein. However, whatever the added iron concentration, a significant fraction of the metal was not retained by the protein and was measured in the filtrate, meaning that some metal ions were not bound by the protein and kept freely available. These results demonstrated that NaCas exhibited better iron-binding properties than BSA.

DISCUSSION

Proteins can prevent or promote lipid oxidation by various mechanisms depending on their physicochemical characteristics (*7*, *9*). In emulsions, lipid oxidation is greatly influenced by electrostatic interactions between ions of transition metals and droplet surface (*36*) but interfacial film thickness, pH, or the presence of solutes in the aqueous phase may also interfere. A better knowledge of the factors that determine the development of lipid oxidation in the emulsions where proteins constitute the stabilizing interface can lead to an optimized use of proteins as food ingredients. Among these factors, the interactions that can take place between the metal ions and the proteins, either at the interface or in the aqueous phase of the emulsions, and their influence on emulsion chemical stability should be investigated.

The experiments were performed in hermetically sealed vials. Considering linoleic acid as the main oxidizable substrate in the oil, the molar ratio for oxygen to substrate was initially about 1:5. Thus, total oxygen amount was the limiting factor for development of oxidation in the vials. Accordingly, oxygen uptake and conjugated dienes achieved a plateau after 30 h irrespective of the emulsifying protein. Maximum measured O_2 uptake accounted for about 80% of the initial amount of O_2.. In fact, this value should be considered as a limit mainly because of the experimental procedure. In the vials, the consumption of oxygen induced by lipid oxidation led to a depression. Hence, when the syringe used to measure O_2 uptake was removed from the vials, some air probably entered in it through the needle until normal pressure was reached. In addition, it has yet been shown that when oxygen concentration is below a certain threshold, it becomes the limiting factor for oxidation. Accordingly, formation of conjugated dienes, chosen as markers of primary products of oxidation, that is, hydroperoxides, achieved simultaneously a plateau. In contrast, secondary products of the reaction because of hydroperoxide decomposition were always produced, even under oxygen-limiting conditions. Concerning the effect of the emulsifying protein, results from measurements of O_2, CD, and volatile compounds at short times (<30 h) were in close agreement. Under the conditions used and at short term aging, that is, as long as oxygen was available, BSA-stabilized emulsions oxidized more slowly than NaCas-stabilized emulsions. When most of the oxygen was consumed, amounts of detected volatiles compounds were similar, irrespective of the emulsifying protein, showing that the decomposition of hydroperoxydes first depended on the total amounts of conjugated dienes produced.

The decrease in pH was also slower in emulsions made with BSA than in emulsions made with NaCas. The decrease of emulsion pH during aging can be attributed to the formation of short-chain aliphatic acids as secondary products of oxidation (*37*). Accordingly, both amounts of most volatile compounds and pH continued to evolve for a long aging time (>50 h, data not shown). Alteration of basic amino acids of the proteins, such as lysyl or histidyl residues, because of their reaction with primary (free radicals or hydroperoxides) or secondary (aldehydes) products of oxidation may also be involved in this pH decrease (*38*).

The water-soluble chelator EDTA greatly delayed lipid oxidation when present in the aqueous phase of the emulsions (**Figure 2** and **Figure 5**). This confirms that cationic metals were involved in the initiation of the reaction. Metal ions are major pro-oxidants in model emulsions and in formulated foods because of their ability to decompose lipid hydroperoxides into free radicals (*2*). Early detection of volatile compounds in the emulsions was also likely related to decomposition of the hydroperoxydes by metal ions (*39*). As compared to storage at ambient temperature, this decomposition was favored by the temperature of incubation (50 °C) used in this work. Mei et al. (*40*) showed that 5 μM ferrous iron added to salmon oil emulsions increased TBARS formation more than 14-fold compared to the control without added Fe^{2+}. Considering the concentration of iron present in the protein powders, the concentrations of iron in the emulsions were at least equal to 2.27 ± 0.02 and 1.34 ± 0.03 μM in the BSA- and NaCas-stabilized emulsions, respectively. This calculation does not take into account the iron that can originate from the oil, chemicals, and emulsification process (homogenizers were in stainless steel and could release some metal traces). Such concentrations were obviously sufficient to provoke early breakdown of the hydroperoxides present as traces in the oil, formation of free radicals, and propagation of the reaction. As the composition of the emulsions differed only by the protein ingredients, the results concerning the greater oxidative stability of BSA-emulsions did not reflect their higher iron concentration. In fact, the above results can be explained by taking into account the respective abilities of BSA and NaCas to bind metal ions. Caseins have metal-chelating properties because of their phosphoseryl groups (*41*). Our results confirm the better iron-binding properties of NaCas compared to BSA (**Table 1**). When added to the aqueous phase of corn oil-in-water emulsions stabilized by a nonionic

surfactant, caseins trapped the iron away from the lipid droplets and reduced metal-induced lipid oxidation. Similar results were found with casein hydrolysates or caseinophosphopeptides (*6*). In the present study, NaCas was the emulsifier and was present both at the interface and in the aqueous phase of the emulsions. Thus, a fraction of the metal ions, chelated by the proteins, was located at the oil/water interface, that is, at a key place to induce the initiation of lipid oxidation. In addition, the negative ζ-potential of the emulsion droplets stabilized by NaCas was 4 times higher than as those stabilized by BSA. This more anionic charge density induced by the NaCas at the interface probably contributed to attract cationic pro-oxidant metals at the oil droplet surface. (*12*, *13*, *36*). Thus, interfacial metal concentration was probably increased when NaCas was used as the emulsifier, which promoted lipid oxidation as compared with BSA.

BSA adsorbed at the interface can also contribute to the relative protection of the emulsions thanks to its free-radical-scavenging properties (*9*). The SH group of BSA can react with free radicals and prevent them from reacting with unsaturated lipids (*10*). This reaction is conditioned by the accessibility of the SH residues to the radicals.

When EDTA was added to the aqueous phase of the emulsions, metals were kept away from the lipid droplets. As previously found (*31*), under these conditions lipid oxidation was delayed irrespective of the stabilizing protein. However, the lag phase was considerably longer in the emulsions made with NaCas than in those made with BSA. Thereafter, the rate of O_2 consumption was similar in the two types of emulsions, showing that the propagation of the reaction was unaffected by the nature of the protein. This result demonstrates that when metal ions were kept away from the unsaturated lipids, caseins adsorbed at the oil droplet interface extended the lag phase and exerted a more efficient antioxidant activity than BSA. This activity is probably due to their capacity to scavenge free radicals (*7*).

Indeed, one cannot exclude that other factors such as interfacial film thickness can also participate in the differences observed (*42*, *43*). As BSA and NaCas have different structures, their packing at the interface can also influence the diffusion of metals, oxygen, and radicals and thereby the kinetics of oxidation.

These results demonstrate that the oxidative stability of protein-stabilized emulsions both depends on the interactions of proteins with transition metal ions and on the antioxidant properties of the emulsifying proteins. At pH = 6.5, that is above the pI of the proteins, the oxidative stability of BSA- and NaCas-stabilized emulsions greatly depends on metal availability. When the metal ions were freely available, the oxidative stability was lower in NaCas- than in BSA-stabilized sunflower o/w emulsions. Under these conditions, the chelating properties of NaCas and electrostatic interactions favored positioning of the metal ions at the interface, a key place to initiate the oxidation reactions. On the contrary, when metal ions were kept in the aqueous phase and thus inactivated by EDTA addition, the free-radical-scavenging potentiality of interfacial proteins was fully expressed.

All food-grade oils and proteins contain traces of iron and other pro-oxidant metal ions. The location of these compounds in emulsions, and more generally in food products, is a critical factor that should be controlled to improve their oxidative stability. Keeping traces of metals in the aqueous phase, that is, away from the lipid droplets, is an effective way to delay lipid oxidation. This suggests that proteins with good chelating properties such as NaCas should not be used as emulsifiers in systems containing highly oxidizable fatty material but as a natural antioxidant added to the aqueous phase after emulsification process.

ABBREVIATIONS USED

BSA, bovine serum albumin;
CD, conjugated dienes;
EDTA, ethylenediamine-tetraacetic acid;
FID, flame ionization detector;
GC, gas chromatography;
NaCas, sodium caseinate;
o/w emulsion, oil-in-water emulsion;
pI, isoelectric point;
SH group, thiol group;
SPME, solid-phase microextraction;
TBARS, thiobarbituric acid reactive substances;
TCD, thermal conductivity detector.

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