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Lipid Oxidation in Algae Oil-in-Water Emulsions Stabilized by Bovine and Caprine Caseins

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Caseins (α_{s1}-, α_{s2}-, and β-casein) are phosphoproteins that are capable of binding transition metals and scavenging free radicals; this property makes them good candidates to be used as natural antioxidants in oil-in-water emulsions. Caprine casein exhibits variability in α_{s1}-casein content generated by genetic polymorphism. This variability in composition could lead to altered antioxidant properties. Thus, the ability of two caprine caseins differing in α_{s1}-casein content to inhibit lipid oxidation in algae oil-in-water emulsions at 5% oil was investigated and compared to bovine caseinate. All caseins inhibited the formation of lipid oxidation at pH 7.0 as determined by lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS). However, caprine caseins were in general more effective inhibitors of lipid oxidation than the bovine caseins, which may be attributed to their altered casein amino acid content and/or metal binding capabilities. The combination of the carotenoids with bovine and caprine caseins was highly effective at repressing oxidation leading to the speculation that the caseins may inhibit the loss of the carotenoids and/or react with and enhance the carotenoid activity; again some differences between bovine and caprine caseins were observed with caprine caseins being slightly more effective in the presence of carotenoids.

KEYWORDS: Bovine casein; caprine casein; emulsion; oxidative stability; carotenoids

INTRODUCTION

Emulsifying properties of proteins in lipid emulsions are primarily due to their hydrophobicity and flexibility (*1*, *2*). However, the absorption behavior of proteins and the conformation of the absorbed proteins at the oil interface are also important parameters in their emulsifying properties (*3*, *4*). Casein and caseinates can assist with emulsifying properties in formulated foods (*5*), and act as interface agents between oil and water and help to form and stabilize emulsions. A casein hydrolysate or casein phosphopeptide isolate is composed of short peptides, and generally does not form large aggregated structures via hydrophobic domains in aqueous solutions. In contrast, the unique assembly of the heterogeneous multisubunit protein complex known as micelles reflects the self-association of α_{s1}-, β- and κ-caseins due to hydrophobic forces (*6*).

Dietary fat contains n-3 and n-6 polyunsaturated fatty acids (PUFA), both of which play important roles in many human biological processes. Docohexaenoic (DHA) and eicosapentaenoic (EPA) fatty acids are n-3 fatty acids found in oily fish and algae. Evidence from epidemiological and clinical studies indicates that the intake of n-3 fatty acids DHA (22:6n-3) and EPA (20:5n-3) even in small amounts is beneficial for reducing the risks of cardiovascular disease and cancer (*7*). The oral administration of fish oil from salmon, lake trout, tuna, and herring also exerts anti-inflammatory effects in patients with rheumatoid arthritis (*8*), psoriasis (*9*, *10*), and ulcerative colitis (*11*). α-Linolenic acid (ALA) is an n-3 fatty acid (18:3n-3) found in the chloroplast of green leafy vegetables such as purslane and spinach and in the seeds of flax, linseed and walnuts. Linoleic acid (18:2n-6) is found in large amounts in all plant seeds with the exception of palm, cocoa, and coconut. Both n-3 and n-6 fatty acids are essential for growth and development, and particularly DHA is important for normal visual and cerebral function in preterm infants (*12*). Human milk is a good source of n-3 fatty acids (*13*), but infants raised on infant formula do not receive enough n-3 fatty acids, as cow's milk contains low amounts of n-3 fatty acids (*14*). The addition of ALA to formula foods may not adequately provide the DHA needs of the premature and possibly the full term infant (*15*, *16*).

Food fortification is a practical approach for increasing the intake of DHA/EPA (*17*). The difficulties encountered when fortifying foods with DHA/EPA are primarily due to the lack of oxidative stability of these PUFA. The n-3 PUFA present in algae and fish oils are readily oxidized to produce rancidity or off-flavor volatiles when exposed to air, light, elevated temperatures, and/or transition metals (*17*). To improve the oxidative stability of algae and fish oils, antioxidants are usually added. The term antioxidant in foods is usually applied to those compounds that interrupt the chain reactions involved in oxidation, but, in a wider sense and according to the classification made by Kochhar and Rossell (*18*), the term may consider five groups: (1) primary

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antioxidants are mainly phenolics, that interrupt the chain of free radicals and among which are found natural and synthetic tocopherols, alkyl gallates, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *ter*-butylhydroquinone (TBHQ); all of these compounds act as donors of electrons; (2) oxygen consumers such as ascorbic acid (vitamin C), ascorbyl palmitate, and erythorbic acid; (3) secondary antioxidants such as dilaurylthiopropianate, which decompose hydroperoxides into stable products; (4) enzymatic antioxidants, which work by eliminating oxygen or highly oxidative species from foods; and (5) chelating or masking agents such as citric acid, amino acids, and ethylenediaminetetracetic acid (EDTA), which form chelates with metallic ions such as copper and iron, thus avoiding their catalytic action on the oxidation of lipids. Most of these compounds exhibit little or no antioxidant activity when used alone, and therefore we should really consider them as synergistic agents of other antioxidants. For instance, chelating agents increase, to a great extent, the antioxidant action of phenolic compounds.

The use of synthetic antioxidants such as BHA, BHT, TBHQ and EDTA is restricted by the FDA because of food safety concerns. Consumers are actively avoiding food products with harmful ingredients. The safety and health benefits of natural antioxidants from fruits and vegetables, such as vitamin C, vitamin E, and the carotenoids, have been reported in numerous studies and recognized by the FDA and many consumers (*19*−*21*). However, information on using natural antioxidants to replace synthetic antioxidants in stabilizing n-3 fatty acids such as DHA and EPA against oxidation is limited. Bovine casein exhibits antioxidant activity in oil-in-water emulsions (*22*, *23*). However, caprine caseins which vary considerably in their relative casein contents (*24*) have not been tested in model systems either for emulsifying capacity or for antioxidant activities. To test the effect of variation in casein composition and to further characterize the antioxidant activity of caseins other than bovine, we have evaluated the antioxidant activity of caprine casein for stabilizing n-3 fatty acids in algae oil-in-water emulsions at 5% oil hopefully to yield smaller droplets with a higher protein to fat ratio to achieve increased stability as food additives. It was also investigated whether the protective antioxidant effect of the carotenoids could be observed in algae oil-in-water emulsions stabilized by caprine casein. Bovine casein was used as control for comparison purposes.

MATERIALS AND METHODS

Materials. Algae oil, a rich source of n-3 fatty acids composed of 3% EPA and 46% DHA, was obtained from Martek Biosciences, Inc. (Boulder, CO). Caromin, a natural mixed 20% carotenoid concentrate complex composed of α-carotene (65 mg/g), *β*-carotene (131 mg/g), lycopene (1.0 mg/g) and other carotenoids (5.0 mg/g), was obtained from Carotech (Perak, Malaysia). All reagents used were of analytical grade, ACS certified or HPLC grade from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Double-deionized water was used in all experiments.

Methods. *Preparation of Bovine and Caprine Caseins*. Caseins were obtained from the milk of a Jersey cow and French-Alpine goats. The samples of the caprine milk were collected from individual French-Alpine animals which were raised at the International Goat Research Center in Prairie View A&M University, TX. The caprine milk caseins were selected based on yielding high and low levels of the α_{s1}-casein component as determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (*24*). Caseins were isolated from 2 L of fresh, uncooled milk to which phenylmethanesulfonyl fluoride (0.1 g/L) was added immediately to retard proteolysis. The milk was centrifuged at 4000*g* for 10 min at room temperature to remove the cream fraction. Skim milk (500 mL) was diluted with an equal volume of distilled water and warmed to 37 °C. Casein was precipitated by gradual addition of 1 N HCl to pH 4.5. The precipitate was homogenized with a hand-held homogenizer (Biospec Products Inc., Bartlesville, OK) at low speed and dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4 °C and centrifuged at 100000*g* for 30 min to remove residual fat. The integrity of the samples was confirmed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), and densitometry was used to assess the relative concentrations of casein components (*25*).

The amino acid content of the bovine and caprine casein samples was measured at The Protein Technologies Laboratory, Department of Entomology, Texas A&M University (College Station, TX).

Iron-Binding Properties of the Bovine and Caprine Caseins. To evaluate the ability of the bovine and caprine caseins to bind soluble iron, mixtures of the casein solution and known amounts of ferrous iron were filtrated through cutoff filters and the unbound metal was quantified (*26*). Ferrous iron was added to the bovine and caprine casein solutions (1 g L^{-1}, pH adjusted to 6.5) at concentrations ranging from 1.0 to 4.0 mg L^{-1}. After 16 h incubation at room temperature, the protein solutions were filtered through disposable cutoff filters (Millipore Corporation, Billerica, MA) to remove free casein and casein-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 phenanthroline reagent (2.5 mM phenanthroline, 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 subtracted from the concentration of added iron to obtain the concentration of bound iron. The experiment was also performed with double distilled water as a control.

Preparation of Emulsions. Oil-in-water emulsions were prepared using different concentrations of algae oil (0.1−5.0%) and bovine casein or caprine casein with high and low α_{s1}-casein (0.1−1.0%) in imidazole-HCl buffer (10 mM, pH 7.0). In emulsions with added carotenoids, the carotenoids (0.01%) were added to the algae oil before emulsification. Thimerosal (1 mM) was added to the emulsions to inhibit microbial growth during storage. Oil-in-water emulsions were made by blending the lipid and aqueous phases for 3 min using a hand-held homogenizer. The coarse emulsion was then homogenized three times at 5000 psi through a high-pressure TC5 homogenizer (Stansted Fluid Power, Harlow, U.K.).

Physical Characterization of Emulsions. The particle size distribution ($d_{3,2}$; μm) of the oil droplets in emulsions was measured at 1, 48, and 96 h after homogenization with a SALD-2101 laser diffraction particle analyzer (Shimadzu, Columbia, MD).

The charge of the emulsion droplets (zeta potential, ζ; mV) was determined by injecting diluted (1:1000, sample:imidazole-HCl buffer, pH 7.0) emulsion into the measurement chamber of a Zetasizer nano ZS (Malvern Instruments, Worcestershire, U.K.).

^{31}P NMR measurements were carried out with a Varian XL-400 spectrometer (Varian Associates, Palo Alto, CA) at 161.8 MHz using a probe (10 mm, 45−165 MHz frequency), a pulse width of 21 μs (57° nutation angle), spectral width 20 kHz, acquisition time 0.82 s and a recycle time of 4.82 s; data were stored in a 32 K point time-domain. About 4 mL of solution and emulsion samples in D_2O (pD 7.2) was run in 10-mm high-resolution NMR tubes (Wilmad, Buena, NJ). NMR measurements were conducted in triplicate at 21 ± 1 °C. The line widths were measured from the resonances at half-height. Spin−lattice relaxation time (T_1) was determined with the inversion method (*27*) using the 180°−τ−90° pulse sequence. The 90° pulse width was 53 s for each 6 s delay time τ. Spin−spin relaxation time (T_2) was determined by the spin−echo method (*28*) using the 90°−τ−180° pulse sequence.

Lipid Oxidation Measurements. Primary oxidation products of lipids in foods can be measured by hydroperoxides, peroxide value or conjugated dienes while the secondary oxidation products are measured by gas chromatography, anisidine value or TBARS (*29*, *30*).

Oxidative stability of emulsion samples (5 mL) was determined by storing them in tightly sealed screw-capped test tubes in the dark at 37 °C for up to 8 days. At each of the storage times a new tube was opened for a different type of sampling, that is, for lipid hydroperoxides and TBARS analyses. The tubes for lipid hydroperoxides and TBARS measurements

were frozen at the end of each sampling time and kept at −80 °C until analysis.

Lipid hydroperoxides were measured with a modified ferric thiocyanate method (*31*, *32*) by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1) by mixing (10 s, 3 times) and isolation of the organic solvent phase by centrifugation at 1000*g* for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/*n*-butanol (2:1), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of a ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4 \cdot 7H_2O$). After 20 min of incubation at room temperature, absorbance was measured at 510 nm with a UV/vis model DU-530 spectrophotometer (Beckman Instruments, Fullerton, CA). Hydroperoxide concentrations were determined using a standard curve from cumene hydroperoxide.

Thiobarbituric acid reactive substances (TBARS) (*33*) were determined by mixing 1.0 mL of emulsion with 2.0 mL of TBA reagent in 15 mL test tubes. TBA reagent was prepared by combining 100 mL of TCA-TBA-HCl solution [15% w/v trichloroacetic acid (TCA) and 0.375% w/v thiobarbituric acid (TBA) in 0.25 M HCl] with 3.0 mL of ethanol (containing 2% BHT). Samples were heated in a boiling water bath for 15 min, cooled to room temperature (10 min), and then centrifuged for 15 min at 3400*g* (Beckman Centrifuge model J2-21, Beckman Instruments Inc., Fullerton, CA). Samples were held at room temperature for 10 min before the absorbance of each sample was measured at 532 nm using a UV/vis model DU-530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). TBARS concentrations were determined using a standard curve from 1,1,3,3-tetraethoxypropane.

β-Carotene Degradation. The quantity of β-carotene in emulsions after storage for specific periods was determined according to the HPLC method described by Bushway (*34*) with few modifications. Briefly, 5 mL of 95% ethanol was added to 2 mL of emulsion at room temperature for 5 min. Five milliliters of tetrahydrofuran (THF) was added and was mixed by vortex for 30 s; the mixture was allowed to stand for 2 min. Three milliliters of double-deionized water was added to the above mixture and mixed by gentle inversion, followed by centrifugation for 10 min at 1000*g* at 15 °C. The upper layer was removed, filtered through a 0.45 μm nylon filter (Millipore Corporation, Billerica, MA) and stored at −20 °C until analysis.

A Waters HPLC system (Waters Associates, Milford, MA) was used with a 5 μm reverse phase column (Nucleosil ODS1, 3.2 × 250 mm) and a UV detector adjusted to 450 nm. The mobile phase used was as follows: 20 parts acetonitrile:75 parts methanol:5 parts THF. Using a flow rate of 0.6 mL/min, 100 μL of sample or standard was injected into the system and the peak was compared with known standards to compare the concentration of β-carotene in the emulsion samples during storage.

Statistical Analysis. In each experiment, the results of triplicate analyses were used to test experimental variables. The data were analyzed by ANOVA using PRO GLM of SAS (version 8.2, SAS Institute, Cary, NC). The least significant test of SAS was used to determine significant differences between means at $p < 0.05$.

RESULTS AND DISCUSSION

The bovine and caprine caseins used in this study had different compositions in terms of α_{s1}-, α_{s2}-, and β-casein content (**Table 1a**). Because of the compositional differences, these milk proteins also exhibited major differences in terms of the content of potential antioxidative amino acids (**Table 1b**). In spite of the fact that β-casein, which has only 5 serine phosphates, predominates in the caprine caseins so that they have a predicted lower casein bound phosphate content, the caprine caseins collectively had higher iron-binding properties (**Table 2**).

Physical Stability of Emulsions. The ingredients in an emulsion partition themselves among the oil, water, and interfacial regions according to their concentration and interactions with the local environment. The location and mass transport of the ingredient molecules within an emulsion have a significant influence on the physical stability of food emulsions. Moreover, isolated β-casein has better emulsifying properties so the caprine caseins richer in this protein (**Table 1a**) might be expected to be better emulsifiers (*35*). For instance, protein concentration is known to influence emulsion droplet size, surface protein concentration, and storage stability (*3*). Thus, five different concentrations of the bovine and caprine caseins (0.1%, 0.2%, 0.5%,1.0%, and 1.5%) were tested at pH 7.0, and the particle size of the algae oil-in-water emulsions (5.0%) stabilized by such milk proteins are shown in **Table 3**. The initial particle size of the bovine and caprine casein-stabilized algae oil-in-water emulsions ranged from 0.25 to 0.43 μm. There were not significant changes in the mean particle diameters of the bovine and caprine casein-stabilized emulsions at all protein concentrations tested (0.1−1.5% casein) during storage at 37 °C for 4 days. The trend (**Table 3**) shows that particle sizes decreased significantly for all caseins at 1.5%. This is in accord with the data which show that above 1.5% the caseinate behaves as a limiting polymer with a Stokes radius of 9.4 nm (*36*), but below 1% sodium caseinate dissociates into smaller aggregates. This leads to mixtures of caseins with smaller sizes, which then may bind independently to the lipid interface (*35*). These concentrations of mixed polymers could accentuate the differences seen in **Table 1a**. In this light the concentrations of caseins used for further testing were ≤0.5%. At pH 7.0, the net charge on the caseins is negative. The charged groups of interfacial proteins present a barrier to the close approach and coalescence of

Table 1. Casein Distribution (**a**) and Content of Potential Antioxidative Amino Acids (**b**) in Bovine and Caprine Caseins[a]

		caprine		
	bovine	high in α_{s1}-casein	low in α_{s1}-casein	SEM
		(**a**) Casein Distribution (g/100 g)		
α_{s2}-casein	12.1 b	9.2 b	29.2 a	2.4
α_{s1}-casein	39.5 a	25.1 b	5.9 c	2.4
β-casein	37.2 b	51.6 a	50.5 a	2.4
κ-casein	11.2	13.8	14.4	2.4
		(**b**) Casein Amino Acid Content (g/100 g)		
histidine	1.27 a	0.79 b	0.77 b	0.02
methionine	1.25 a	0.81 b	0.82 b	0.12
phenylalanine	2.02 a	1.31 b	1.29 b	0.09
proline	3.84	3.18	3.19	0.2
tryptophan	0.90	0.87	0.88	0.02
tyrosine	4.02	3.96	3.97	0.13

[a] Means in the same row with different letters a−c are different ($p < 0.05$). Data are the mean of triplicate measurements.

Table 2. Retention of Ferrous Iron by Solutions of Bovine and Caprine Caseins[a]

			caprine	
total iron (mg/L)	control	bovine (1 g/L)	high α_{s1}-casein (1 g/L)	low α_{s1}-casein (1 g/L)
1.0	0.01 ± 0.02 a	0.61 ± 0.01 b	0.67 ± 0.05 c	0.65 ± 0.02 c
2.0	0.02 ± 0.01 a	1.15 ± 0.02 b	1.42 ± 0.02 c	1.44 ± 0.01 c
4.0	0.13 ± 0.09 a	2.42 ± 0.02 b	2.54 ± 0.04 c	2.51 ± 0.01 c

[a] Bound iron, mg/L. Values represent the mean ± SD. Means in the same row with different letters a−c are different ($p < 0.05$).

Table 3. Mean Particle Size (μm) of Algae Oil-in-Water Emulsions (5%) Stabilized by Different Concentrations of Bovine and Caprine Caseins (0.1−1.5%) during Storage[a]

storage time (h)	0.1%	0.2%	0.5%	1.0%	1.5%	SEM
			Bovine			
1	0.42 a	0.40 ab	0.38 ab	0.38 ab	0.33 b	0.03
48	0.40 a	0.38 ab	0.40 a	0.38 ab	0.30 b	0.03
96	0.43 a	0.39 a	0.37 a	0.39 a	0.28 b	0.03
			Caprine High α_{s1}-Casein			
1	0.43 a	0.41 a	0.41 a	0.39 a	0.30 b	0.03
48	0.41 a	0.38 ab	0.40 a	0.38 ab	0.31 b	0.03
96	0.41 a	0.37 ab	0.39 ab	0.39 ab	0.32 b	0.03
			Caprine Low α_{s1}-Casein			
1	0.42 a	0.38 a	0.38 a	0.39 a	0.29 b	0.03
48	0.40 a	0.39 a	0.42 a	0.40 a	0.25 b	0.03
96	0.40 a	0.39 a	0.41 a	0.37 ab	0.30 b	0.03

[a] Means with the same letters a or b are not different ($p < 0.05$). Data are the mean of triplicate measurements.

neighboring droplets (*3*). These results show the ability of bovine and caprine caseins in producing smaller lipid droplets which make the emulsions more stable and perhaps useful, *per se*, as food ingredients.

The high number of negatively charged groups may be partly responsible for the observed stability of the algae oil-in-water emulsions made with the bovine and caprine caseins at all protein concentrations tested. Accordingly, the droplet charge (zeta potential, ζ) of the emulsions at all protein concentrations tested (0.1−1.5% casein) ranged from −41.2 mV to −44.3 mV for bovine, −33.1 mV to −37.4. mV for caprine casein high in α_{s1}-casein, and −35.0 mV to −38.1 mV for caprine casein low in α_{s1}-casein during the course of the study (data not shown). Here the lower phosphoserine contents of the caprine caseins do yield lower zeta potentials. The highly charged surfaces present in the bovine and caprine caseins in emulsions at pH 7.0 reduce interactions between droplets through electrostatic repulsions.

Particle size of a food emulsion is also influenced by the concentration of the oil used (*3*). The particle size of the emulsions stabilized with 0.5% bovine and caprine caseins increased with increasing algae oil content from 0.1 to 1.0% (**Table 4a**). Particle size of the emulsions was 0.22 μm, 0.20 μm, and 0.24 μm for bovine casein, caprine casein high in α_{s1}-casein and caprine casein low in α_{s1}-casein, respectively, at 0.1% of algae oil concentration. However, the particle sizes increased to 0.67 μm, 0.65 μm, and 0.68 μm for bovine casein, caprine casein high in α_{s1}-casein and caprine casein low in α_{s1}-casein, respectively, when the concentration of algae oil was increased from 0.1% to 1.0%. Note however, that at 5% oil the sizes dropped to lower values (**Table 3**). It would appear that for 0.5% caseinate the 5% oil would be maximum in producing small stable emulsions for use as food ingredients. **Table 4b** presents the specific surface area derived from the particle size distributions (data not shown) against algae oil content of emulsion. The specific surface area formed by the bovine and caprine caseins decreased with increasing algae oil content as a result of formation of larger droplet size of emulsions. No statistically significant differences as to surface areas were found when the bovine and caprine caseins were treated with increasing amounts of the algae oil. These data indicate that the droplet size and surface areas of the casein-stabilized emulsions are closely related to the ratio of oil/protein in the emulsion.

Table 4. Mean Particle Size (**a**) and Surface Area (**b**) of Algae Oil-in-Water Emulsions Stabilized by Bovine and Caprine Caseins (0.5%) as a Function of Algae Oil Concentration (0.1−1.0%)[a]

		caprine		
algae oil (%)	bovine	high α_{s1}-casein	low α_{s1}-casein	SEM
		(**a**) Mean Particle Size (μm)		
0.1	0.22 c	0.20 c	0.24 c	0.03
0.2	0.25 c	0.24 c	0.27 c	0.03
0.5	0.36 b	0.39 b	0.38 b	0.03
1.0	0.67 a	0.65 a	0.68 a	0.03
		(**b**) Surface Area (m^2/g)		
0.1	17.3 a	16.8 a	17.1 a	0.18
0.2	14.0 b	13.5 b	13.7 b	0.18
0.5	8.7 c	8.4 c	8.6 c	0.18
1.0	6.9 d	6.7 d	6.7 d	0.18

[a] Means with different letters a−d are different ($p < 0.05$). Data are the mean of triplicate measurements.

Emulsifiers, i.e., casein and caseinates, have the ability to form and stabilize emulsions by being absorbed to the oil-in-water interface during homogenization, reducing the interfacial tension by an appreciable amount, thus preventing droplet coalescence from occurring during homogenization (*3*, *5*). The interfacial activity of proteins is due to the fact that they have both hydrophilic and hydrophobic regions distributed along their backbones. Most proteins have significant numbers of exposed nonpolar amino acid side groups (*37*). Therefore, the major driving force for absorption of proteins to oil−water interfaces is the hydrophobic effect. When a protein (BSA) is absorbed to an interface, it may or may not adopt a conformation where the nonpolar groups are located in the oil phase and the hydrophilic groups are located in the aqueous phase. Adsorption also reduces the contact area between the oil and water molecules at the oil−water interface, which lowers the interfacial tension. In addition the nonabsorbed protein in the continuous phase can influence emulsion stability. So the caseins because of their hydrophobicity, high flexibility, and high degree of adsorption are usually better emulsifying agents (*35*).

Figure 1 shows ^{31}P NMR spectra of caprine casein low in α_{s1}-casein (0.5%) in solution and in emulsions at 0.2, 0.5, and 1.0% algae oil. The narrow ^{31}P NMR spectrum observed in an aqueous solution containing the caprine casein low in α_{s1}-casein (**Figure 1**) represents α_{s1}-casein phosphates originating from, or at least present in, very similar environments as a large fraction of the phosphorylated serine (SerP) residues in the α_{s1}-, α_{s2}-, and β-caseins at pH 7.0 (**Table 1a**). As the concentration of the algae oil is increased, the broadening of the ^{31}P NMR spectra in algae oil-in-water emulsions suggests the possibility of a conformational change of the bovine and caprine caseins induced by their interaction with the oil phase and increased helical structure has been noted for β-casein (*38*, *39*) when bound to surfaces. Alternatively, being bound to the droplet causes the casein molecule to sense the motion of the large particle and the line width increases (*40*). Note that for these concentrations the particle sizes do increase in proportion to the line broadening (**Table 4a**). The conformation that the bovine and caprine caseins adopt at an oil−water interface seems to depend on their molecular structure and interactions. As noted above the properties of the bovine and caprine caseins produce good emulsions which have a tendency to form thin and compact membranes that have high viscoelasticities.

The motional properties of the SerP residues in α_{s1}-, α_{s2}-, and β-caseins of bovine and caprine casein solutions and emulsions

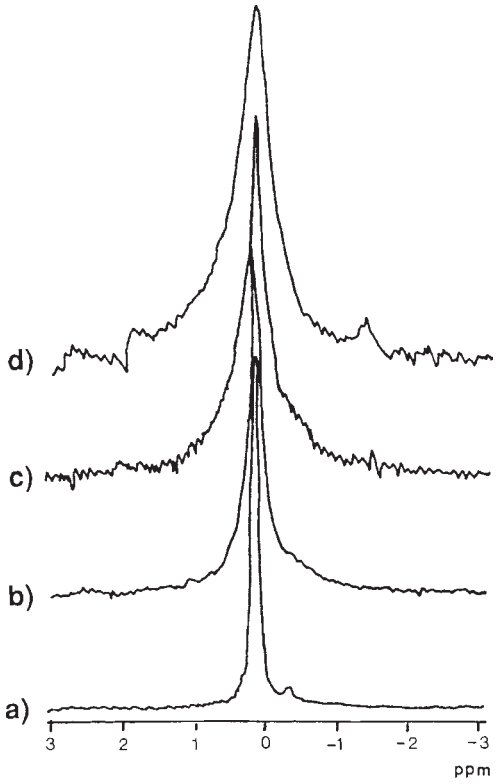

Figure 1. ^{31}P NMR spectra of caprine casein low in α_{s1}-casein in an aqueous dispersion and emulsions: (**a**) the solution of 0.5% protein. The algae oil-in-water emulsions were with 0.2% (**b**), 0.5% (**c**), and 1.0% (**d**) algae oil concentrations in 0.5% protein.

at 0.5% concentration of casein containing different concentrations of algae oil (0.2%, 0.5%, and 1.0%) were also evaluated (**Table 5**). The ^{31}P NMR T_1 relaxation times of the SerP residues in α_{s1}-, α_{s2}, and β-caseins of bovine and caprine casein-stabilized emulsions were markedly decreased from 1.49 to 0.33 s for bovine casein, 1.51 to 0.29 s for caprine casein high in α_{s1}-casein, and 1.54 to 0.25 s for caprine casein low in α_{s1}-casein (**Table 5**). The ^{31}P NMR T_2 relaxation times were also decreased in the casein-stabilized emulsions as compared with the casein solutions (**Table 5**). All of this indicates the ability of the bovine and caprine caseins to adsorb to the oil−water interface during homogenization. Our results are in agreement with the ^{31}P NMR work obtained with bovine caseinates in triacylglycerol-in-water emulsions (*41*). However, our results indicate that the caprine caseins absorbed to the algae oil had more restrictions of molecular motions in comparison with those of the bovine casein in algae oil-in-water emulsions (**Table 5**). The caprine caseins seem to exhibit more hydrophobic domains, and thereby, have more affinity for the oil−water interface. The phosphoprotein β-casein is a major constituent of the caprine caseins (**Table 1a**), and the outstanding feature of β-casein is its highly hydrophobic character (*42*) which enables it to insert its hydrophobic sites compactly into the lipid and present its hydrophilic end on the surface. This restricts motion relative to the solution state (*41*). It has been suggested that the native states of the α_{s1}-, α_{s2}-, β- and κ-caseins are the states in which they exist when fully immersed in the casein aggregates or in casein micelles with strong hydrophobic interactions occurring among the caseins. In contrast monomeric forms of caseins are classified as natively unfolded coils (*43*, *44*). However, upon self-association, three of the caseins become more compact with β and κ classified as molten globules and α_{s2} becoming a premolten globule. Interestingly, α_{s1}-casein remains

Table 5. ^{31}P-NMR Line Widths and Relaxation Times of Bovine and Caprine Caseins (0.5%) in Solution (0% Algae Oil) and Emulsions (0.2−1.0% Algae Oil)[a]

algae oil (%)	line widths (Hz)	T_1 (s)	T_2 (ms)
	Bovine		
0	21.3 ± 1 fg	1.49 ± 0.06 a	31.1 ± 3 a
0.2	25.1 ± 3 ef	1.38 ± 0.01 b	28.0 ± 5 b
0.5	48.6 ± 2 d	0.91 ± 0.04 d	12.4 ± 2 d
1.0	93.0 ± 3 b	0.33 ± 0.01 e	9.3 ± 1 e
	Caprine High α_{s1}-Casein		
0	18.4 ± 3 g	1.51 ± 0.02 a	27.5 ± 2 b
0.2	22.9 ± 1 f	1.27 ± 0.05 c	21.8 ± 1 c
0.5	53.7 ± 1 c	0.86 ± 0.03 d	10.0 ± 5 de
1.0	102 ± 5 a	0.29 ± 0.01 e	9.5 ± 3 def
	Caprine Low α_{s1}-Casein		
0	17.6 ± 2 g	1.54 ± 0.05 a	28.2 ± 6 b
0.2	28.3 ± 7 e	1.31 ± 0.03 bc	22.7 ± 4 c
0.5	51.5 ± 4 ed	0.83 ± 0.02 d	11.3 ± 1 de
1.0	98.3 ± 1 a	0.25 ± 0.02 e	6.5 ± 5 f

[a] Values represent the mean ± SD. Means in the same column with different letters a−g are different ($p < 0.05$).

an unfolded coil in the aggregated state, but acts to reduce the polymerization of the other caseins (*6*). It can be speculated then that low weight casein aggregates, when inserted into the lipid droplets, become more compact as in the case for β-casein. All of these data show that at 0.5% the caseins are highly absorbed to the lipid droplets, and that the maximal lipid content (**Tables 3** and **4a**) is 5% with a protein:oil ratio of 1:10 for the desired particle sizes as food additives.

Oxidative Stability of Emulsions. *Effects of the Bovine and Caprine Caseins*. The most effective means in controlling lipid oxidation in emulsions is to use a combination of antioxidants. Chelating agents are not antioxidants but are often used in combination with well-known antioxidants such as tocopherols, ascorbyl palmitate, and the carotenoids. Chelating agents form complexes with pro-oxidative metal ions such as iron and copper. Caseins have SerP groups capable of binding transition metals (*22*, *23*). However, the type and proportion of the phosphoproteins α_{s1}-, α_{s2}- and β-caseins in whole casein from bovine and caprine milks varies (**Table 1a**), which may lead to differences in chelating activity and, thereby, affect the oxidative stability of oil-in-water emulsions. In addition the motional differences found in the NMR work argue for the possibility of subtle surface coating.

The bovine and caprine caseins at different concentrations (0.1−0.5%) were able to significantly inhibit lipid hydroperoxide formation in algae oil-in-water emulsions at pH 7.0 stored for 7 days at 37 °C (**Table 6**). The antioxidant effect exhibited by the bovine and the two caprine caseins in terms of hydroperoxide formation was equally effective at all three concentrations of casein used. There were no significant differences among the bovine casein, the caprine casein high in α_{s1}-casein, and the caprine casein low in α_{s1}-casein in stabilizing the algae oil-in-water emulsions against oxidation in terms of hydroperoxide formation (**Table 6**).

The antioxidant effect, as measured by TBARS formation, exhibited by the bovine and caprine caseins in algae oil-in-water emulsions (**Table 7**) showed the existence of an optimal protein concentration in improving antioxidant activity of the bovine and the two caprine caseins at 0.5%. There were no significant differences among the bovine, the caprine casein high in α_{s1}-casein, and the caprine casein low in α_{s1}-casein in stabilizing

Table 6. Ability of Bovine and Caprine Caseins (0.1-0.5%) with and without the Carotenoids (0.01%) To Inhibit Lipid Hydroperoxide Formation in Algae Oil-in-Water Emulsions (5%) at pH 7.0 Stored for 7 Days at 37 °C[a]

	hydroperoxide (mM)	
treatments	without carotenoids	with carotenoids
+0.1% bovine	66.6 ± 1.8 abg	6.9 ± 0.13 ah
+0.1% caprine high α_{s1}-casein	68.5 ± 1.8 ag	6.5 ± 0.13 bh
+0.1% caprine low α_{s1}-casein	69.0 ± 1.8 ag	6.3 ± 0.13 bch
+0.2% bovine	61.5 ± 1.8 bcg	6.8 ± 0.13 abh
+0.2% caprine high α_{s1}-casein	60.9 ± 1.8 cg	6.4 ± 0.13 bch
+0.2% caprine low α_{s1}-casein	63.0 ± 1.8 bcg	6.4 ± 0.13 bch
+0.5% bovine	63.7 ± 1.8 abcg	6.6 ± 0.13 abh
+0.5% caprine high α_{s1}-casein	63.7 ± 1.8 abcg	6.2 ± 0.13 bch
+0.5% caprine low α_{s1}-casein	62.3 ± 1.8 bcg	6.1 ± 0.13 ch

[a] Values represent the mean ± SD. Means in the same column with different letters a−c are different ($p < 0.05$). Means in the same row with different letters g or h are different ($p < 0.05$).

Table 7. Ability of Bovine and Caprine Caseins (0.1−0.5%) with and without the Carotenoids (0.01%) To Inhibit TBARS Formation in Algae Oil-in-Water Emulsions (5%) at pH 7.0 Stored for 7 Days at 37 °C[a]

	TBARS (μM)	
treatments	without carotenoids	with carotenoids
+0.1% bovine	236.3 ± 4.7 ae	66.8 ± 1.5 ad
+0.1% caprine high α_{s1}-casein	236.5 ± 4.7 ae	62.6 ± 1.5 abd
+0.1% caprine low α_{s1}-casein	228.5 ± 4.7 abe	61.8 ± 1.5 bd
+0.2% bovine	223.9 ± 4.7 abe	62.9 ± 1.5 abd
+0.2% caprine high α_{s1}-casein	217.6 ± 4.7 be	59.7 ± 1.5 bd
+0.2% caprine low α_{s1}-casein	217.3 ± 4.7 be	59.2 ± 1.5 bd
+0.5% bovine	213.2 ± 4.7 be	55.4 ± 1.5 cd
+0.5% caprine high α_{s1}-casein	213.6 ± 4.7 be	52.8 ± 1.5 cd
+0.5% caprine low α_{s1}-casein	214.4 ± 4.7 be	53.7 ± 1.5 cd

[a] Values represent the mean ± SD. Means in the same column with different letters a−c are different ($p < 0.05$). Means in the same row with different letters d or e are different ($p < 0.05$).

the algae oil-in-water emulsions against oxidation in terms of TBARS formation at 0.2% protein concentration used. An antioxidant enhancing effect was observed for the caprine casein low in α_{s1}-casein compared with the bovine casein and caprine casein high in α_{s1}-casein at 0.1% protein concentration (**Table 7**). The caprine casein low in α_{s1}-casein contains a high amount of α_{s2}-casein (**Table 1a**). This phosphoprotein contains a higher percent of positive charge (*44*). Although we could not directly compare the data from aqueous solutions and emulsions because the structures of the bovine and caprine caseins are different in the two systems (e.g., the form found in an aqueous solution vs surface inserted, perhaps more compact with increased structure form in emulsions), it is hypothesized that the positively charged clusters of the caprine casein low in α_{s1}-casein may keep solubilized iron (or other cations) in the aqueous phase away from the lipid droplets. Therefore, the observations that emulsions stabilized by caprine casein high and low in α_{s1}-casein (i.e., 0.1% casein concentration) had similar levels of hydroperoxide (**Table 6**) but the emulsions stabilized by the caprine casein low in α_{s1}-casein had decreased TBARS formation (**Table 7**) suggest a slower decomposition of lipid hydroperoxides perhaps favored by the lower amounts of iron ions around the lipid droplet. Note that, at all protein concentrations, particle sizes were equivalent (**Table 3**) but the NMR parameters of **Table 5** had suggested that the caprine caseins had significantly more line broadening than the bovine casein at higher oil contents, thus more insertion into the particles is possible at lower protein concentrations, but the differences in TBARS disappear at 0.5%. The results presented in **Table 6** further show that in a casein-stabilized model emulsion system at pH 7.0 both bovine and caprine caseins inhibited lipid hydroperoxide formation, most likely by their ability to bind pro-oxidant trace metal ions (e.g., iron). The SerP residues present in β-casein bind iron (*45*−*47*). Binding of iron is very strong, about 100 times stronger than calcium binding (*46*). Our results confirm the better iron-binding properties of the caprine caseins compared to the bovine casein (**Table 2**). Binding of iron to the SerP residues of β-casein is expected to increase the oxidative stability of algae oil-in-water emulsions stabilized by bovine and caprine caseins (**Table 6**). However, the protective effect of the caprine caseins added to the emulsions was not different from those emulsions protected by the bovine casein (**Table 6**).

Moreover, the caprine caseins have lower concentrations of certain antioxidative amino acids than the bovine casein (**Table 1b**). Tyrosine and histidine are responsible for the free-radical scavenging effect of bovine casein (*22*). On the other hand, methionine residues in bovine β-casein are the preferred targets for oxidation (*48*). It should be noted here that β-casein is the most prominent radical scavenger in bovine milk (*49*). The high radical scavenging activity of the caseins has been ascribed to their constituent amino acids and their natively unfolded structures (*43*, *44*). These findings are based on the scavenging of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals ($ABTS^{\bullet+}$) in the Trolox equivalent antioxidant capacity (TEAC) assay and peroxyl radicals formed from cleavage of 2,2′-azobis-(2-amidinopropane) (AAPH) in the oxygen radical absorbance capacity (ORAC) fluorometric assay (*49*). The radical scavenging activity of β-casein in caprine milk remains to be elucidated. Proline concentration in the bovine and caprine casein preparations was similar (**Table 1b**), and proline residues have also been reported as target amino acids for oxidative damage in metal-catalyzed free radical generating systems containing collagen and other polypeptides (*50*). The lipid hydroperoxide data presented in **Table 6** suggest that the antioxidant activity of the bovine and caprine caseins can be interpreted on the basis of their ability to chelate pro-oxidant metal ions (e.g., iron) as well as scavenging free radicals. However, the caprine caseins might also be less sensitive to free-radical induced oxidation due to their lower content of certain antioxidative amino acids (**Table 1b**) known to be the target of oxidation (*22*). When lipid hydroperoxides decompose, reactive free radicals are formed. These free radicals can facilitate further oxidation (**Table 7**). The bovine and caprine caseins inhibited lipid oxidation for 7 days at 37 °C as determined by both hydroperoxide and TBARS formation (**Table 6** and **Table 7**).

Our results revealed that lipid oxidation in the algae oil-in-water emulsions is also time-dependent (**Figure 2** and **Figure 3**). At 0.5% protein concentration and 5% algae oil, lipid oxidation was increased after 1 day of storage in all emulsions (**Figure 2** and **Figure 3**). From day 1 to day 8 both the bovine and caprine casein-stabilized emulsions had significantly ($p < 0.05$) more hydroperoxide (**Figure 2**). Likewise, the bovine and caprine casein-stabilized emulsions had significantly ($p < 0.05$) more TBARS throughout the storage period (**Figure 3**).

Effects of the Carotenoids. In addition to serving as a precursor of retinol (vitamin A), β-carotene is a potent quencher of singlet oxygen and an antioxidant. Singlet oxygen is a major pro-oxidant in oils, and, therefore β-carotene and other carotenoids have the ability to enhance the oxidative stability of fatty acids (*51*, *52*). After 1 day of storage, significant differences were observed in the quantities of hydroperoxide and TBARS formed between all treatments containing the carotenoids as compared with those treatments without the carotenoids (**Figure 2a** and

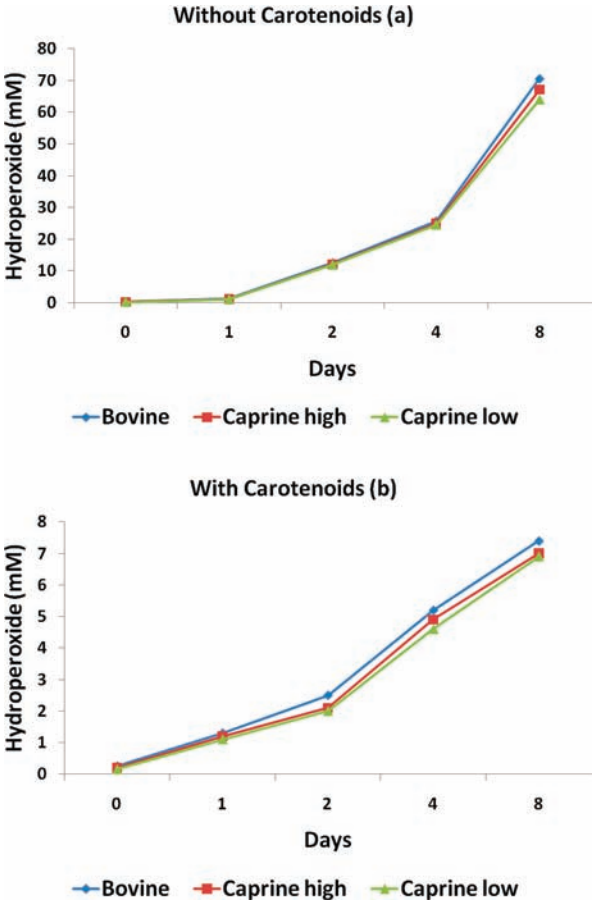

Figure 2. Time dependent hydroperoxide formation (mM) in algae oil-in-water emulsions (5%) at pH 7.0 stored at 37 °C in the presence of bovine and caprine caseins (0.5%) (**a**) without carotenoids and (**b**) with carotenoids (0.01%). Values represent the mean of three trials. The bovine values on the curves without carotenoids are significantly different than the two caprine values ($p < 0.05$), but the two caprine values while different show only a trend for lower values for the caprine casein low in α_{s1}-casein.

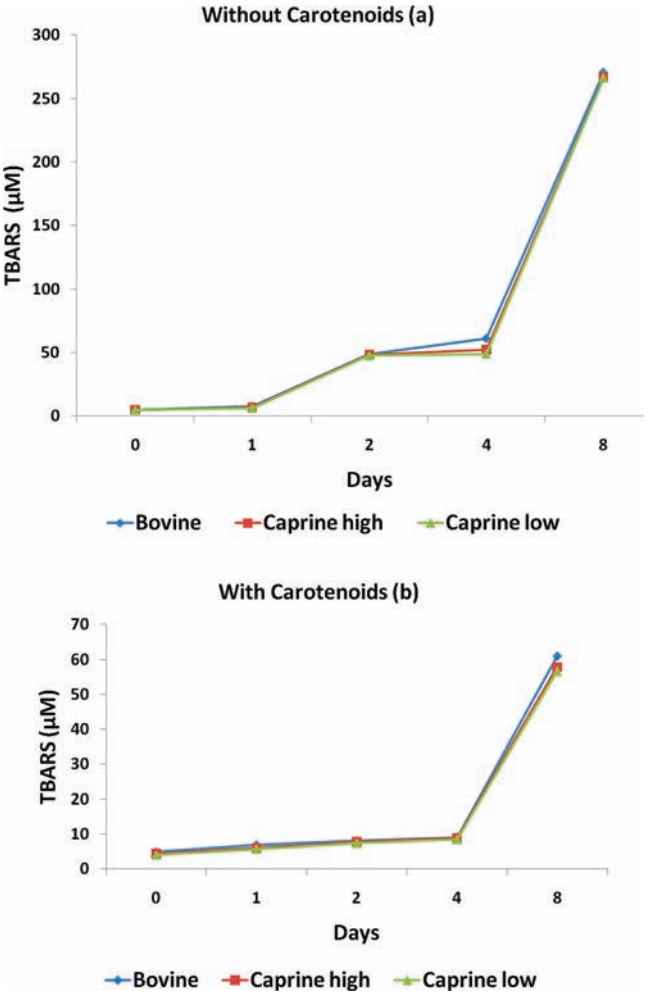

Figure 3. Time dependent TBARS formation (μM) in algae oil-in-water emulsions 5% (pH 7.0) stored at 37 °C in the presence of bovine and caprine caseins (0.5%) (**a**) without carotenoids and (**b**) with carotenoids (0.01%). The bovine values on the curves without carotenoids are significantly different than the two caprine values ($p < 0.05$), but the two caprine values while different show only a trend for lower values for the caprine casein low in α_{s1}-casein.

Figure 3a). These differences in the quantities of hydroperoxide and TBARS formed indicate a highly protective effect from the carotenoids when used in the casein-stabilized emulsions. It should be noted here that the content of β-carotene in the casein-stabilized emulsions containing the carotenoids remained practically stable during the study period (data not shown). The integrity of the other carotenoids i.e., α-carotene, lycopene, is suggested but not proven by the enhanced oxidative stability observed for the casein-stabilized emulsions with the carotenoids as compared with those casein-stabilized emulsions without the carotenoids.

The carotenoids at a concentration of 0.01% inhibited lipid hydroperoxide and TBARS formation in algae oil-in-water emulsions stabilized by bovine and caprine caseins (**Figure 2b** and **Figure 3b**). The carotenoids, in particular β-carotene, impart a degree of oxidative stability to emulsions (*53*) and seem to work synergistically with the bovine and caprine caseins added to the algae oil-in-water emulsions (**Figure 2b** and **Figure 3b**). The lipid hydroperoxide and TBARS formed in the casein-stabilized emulsions containing the carotenoids were similar with no statistical differences between the treatments. Thus it is observed that there exists a physical interaction between the caseins and the carotenoids. In this regard β-casein can bind the large hydrophobic probe ANS (*54*) and vitamin D (*55*). In fact, one report notes that vitamin A is stabilized in processed foods by caseins (*56*).

Our findings in the present study show that the addition of bovine and caprine caseins to algae oil-in-water emulsions reduces the amount of lipid hydroperoxides and TBARS. The differences in the oxidative stability of the algae oil-in-water emulsions stabilized by the bovine and caprine caseins could be related to differences in their ability to chelate metal ions away from the oil droplet, differences in their content of antioxidative amino acids that can scavenge free radicals and/or the sensitivity of such antioxidative amino acids toward free-radicals which induce oxidation. Differences between the bovine and caprine caseins in oxidative stability could be due to differences in how these bovine and caprine milk proteins impact the thickness or packing of the emulsion droplets at the interface. The antioxidant-enhancing effect of the carotenoids in combination with bovine and caprine caseins contributed to the stability of algae oil-in-water emulsions significantly and is a practical approach to utilize the potentials of these dairy ingredients as natural antioxidants in food emulsions.

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