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Investigating the Hydrogen Peroxide Quenching Capacity of Proteins in Polyphenol-Rich Foods

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ABSTRACT: Polyphenols are widely regarded as antioxidants, due in large part to their free radical scavenging activities and their ability to disrupt radical chain propagation. However, recent studies have demonstrated that the oxidation of some polyphenolic compounds, such as the tea-derived compound (−)-epigallocatechin-3-gallate (EGCG), results in the generation of reactive oxygen species that can potentially compromise the oxidative stability of food lipids under some conditions. In this present study, the rate of hydrogen peroxide (H_2O_2) generation and its stability, resulting from EGCG oxidation in Tween 80- and sodium caseinate-stabilized oil-in-water (O/W) emulsions in the presence of iron (25 μM Fe^{3+} from $FeCl_3$), were examined. Observed H_2O_2 levels in protein-stabilized emulsions were significantly lower across all treatments as compared to surfactant-stabilized emulsions. The lower observed H_2O_2 concentrations seen in the protein system are likely due to the antioxidant effects of the added proteins, which either prevented the generation of or more likely scavenged the peroxide. All protein-stabilized emulsions containing EGCG showed increases in carbonyl concentrations, a marker of protein oxidation, throughout the study. The H_2O_2 scavenging activity of aqueous phase and interfacial caseinate and whey protein isolate (WPI) was also evaluated. Both proteins showed concentration-dependent scavenging of H_2O_2 with caseinate displaying significantly higher scavenging abilities at all concentrations. These results suggest that food proteins may play an important role in mitigating the pro-oxidant effects of polyphenols.

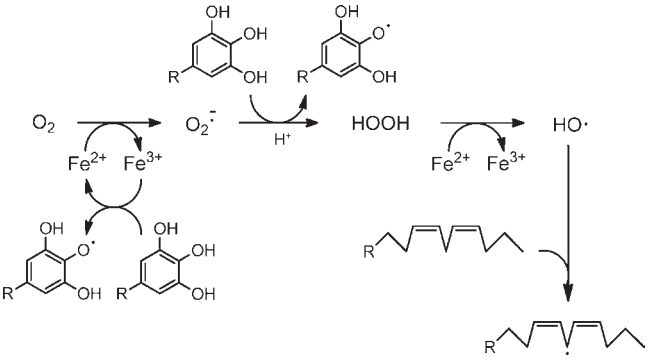
KEYWORDS: Food oil-in-water emulsions, bioactive polyphenols, (−)-epigallocatechin-3-gallate (EGCG), hydrogen peroxide, protein oxidation

INTRODUCTION

Dietary polyphenols have been associated with a decreased risk of age-related diseases in recent years, making them attractive functional food ingredients. However, the incorporation of these compounds into formulated foods can be challenging due to their susceptibility to oxidative deterioration. Furthermore, nonenzymatic polyphenol oxidation is coupled with oxygen reduction,[1] resulting in reactive oxygen species (ROS) formation that can potentially promote the oxidation of other food ingredients (Scheme 1). While the pro-oxidant activity of low (i.e., micromolar concentrations) of flavonoids, particularly the catechins, has been reported in lipid foods,[2] the mechanism underlying this activity is still unclear. Furthermore, the role of H_2O_2 generation (e.g., factors affecting production, reduction, and fate) in promoting oxidation in these systems has been largely ignored.

(−)-Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in tea (*Camellia sinensis*) with demonstrated health benefits that include antiobesigenic and cancer preventative activities.[3−5] Many of these benefits have previously been attributed to EGCG's antioxidant capacity,[4,6] which stems from its ability to quench radical species.[7] Catechins, such as EGCG, are thought to behave classically as a chain-breaking antioxidant by hydrogen atom transfer (HAT) and/or single electron transfer (SET) reactions,[8] thereby inhibiting peroxidation reactions in lipid foods. Polyphenolic compounds are also known to bind metal ions, which may also account for some of EGCG's antioxidant activity.[9] EGCG is known to preferentially bind transition metal ions (e.g., ferric ions; Fe^{3+}) with a 1:2 metal:ligand ratio[10] or a 2:1 metal:ligand ratio when examined with a molar excess (pseudo first order conditions) of Fe^{3+}, with complexation occurring at both B and D rings.[11]

Scheme 1. Proposed Mechanism of Polyphenol-Mediated Lipid Oxidation[a]



[a] For simplicity, the oxidation of the galloyl group is depicted.

Most dietary polyphenols, including EGCG, are readily oxidized in foods, especially at neutral and alkaline pH values. These "autoxidative" reactions, which actually appear to be catalyzed by transition metals in most cases (Scheme 1), are coupled to the

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reduction of oxygen to ROS, namely, superoxide, or its protonated version (hydroperoxyl radicals) and, eventually, H_2O_2.[12−14] While H_2O_2 by itself is not a potent oxidant ($E^{0'}$ = 320 mV at pH 7.0 for the H_2O_2, H^+/H_2O, $^{\bullet}OH$ couple), it is easily reduced to yield highly reactive hydroxyl radicals ($E^{0'}$ = 2310 mV at pH 7.0 for the $\bullet OH,H^+/H_2O$ couple) by transition metal catalysts. This reaction is classically referred to as the Fenton reaction and results in the production of highly oxidizing hydroxyl radicals (•OH) in foods due to the ubiquity of trace levels of iron and copper. The reactivity of ·OH radicals is such that they are thought to react with organic matter (e.g., lipids, proteins, and DNA) at diffusion-limited rates.[15−17]

In theory, classical chain-breaking antioxidants are ill-equipped to scavenge $^{\bullet}OH$ radicals given their reactivity. A more effective approach would be to prevent the formation of these radicals by scavenging their parent peroxides. Proteins have been shown to quench peroxides under biological conditions and, as such, may be effective preventative antioxidants in polyphenol-containing foods. In particular, methionine residues are known to reduce H_2O_2 and lipid hydroperoxides to nonreactive hydroxides by two electron processes.[18−20] This may account for some of the antioxidant activity of proteins observed in lipid dispersions[21,22] and may be a viable mechanism for scavenging polyphenol-generated H_2O_2. The sulfhydryl groups of cysteine residues may also reduce H_2O_2 by a similar mechanism. Aoshima et al. showed that H_2O_2, produced in an aqueous food system by polyphenol oxidation, was effectively reduced in the presence of L-cysteine or glutathione.[23] Thus, the observation that proteins scavenge H_2O_2 may explain why tea catechins show antioxidant activity in protein-rich multiphasic foods, such as meat systems.[24−28] It is conceivable that the radical scavenging properties of polyphenols (e.g., their ability to disrupt lipid peroxidation by quenching lipid radicals) are revealed only once H_2O_2 is removed.

Sodium caseinate and whey protein isolate (WPI) were evaluated for their ability to consume H_2O_2 in this study. Casein and WPI are common functional ingredients in foods that are often utilized for their ability to stabilize emulsions. However, these proteins also exhibit antioxidant activity in foods,[29−31] although their mode of protective action appears to be multifaceted and complex. Sodium caseinate is a composite of proteins consisting of α_{s1}- and α_{s2}-caseins, β-casein, and κ-casein, with an average ratio of 11:2.7:10:4.[32] α_{s1}-Casein, α_{s2}-casein, β-casein, and κ-casein possess five, four, six, and two methionine residues, respectively. Caseinate's antioxidant activity has been attributed to metal chelation by phosphoseryl groups on α_{s2}- and β-casein, as well as radical scavenging by its constituent amino acids. For example, caseinate hydrolysates and enriched caseinophosphopeptides were able to reduce lipid hydroperoxide concentrations in corn oil-in-water emulsions.[31] As is the case with caseinate, whey protein is not a single protein but rather is comprised principally of β-lactoglobulin and α-lactalbumin at 51 and 20%, respectively, with other minor proteins, such as bovine serum albumin (6%) and immunoglobulins (11%), present as well. β-Lactoglobulin, α-lactalbumin, and bovine serum albumin contain four, one, and four methionine residues, respectively. Much of WPI's antioxidant activity has been attributed to the free sulfhydryl groups of β-lactoglobulin, of which it has only one, with the others participating in disulfide bonds. In one study, sulfhydryl groups were blocked in high molecular weight fractions of WPI, causing a 60% decrease in antioxidant activity in a model food lipid system.[33] The importance of solvent accessibility of these amino acids also appears to be important as β-lactoglobulin hydrolysates, and thermally treated β-Lg inhibited the formation of lipid oxidation products in an oil-in-water emulsion to a greater degree than native β-lactoglobulin.[34,35]

The objective of this study was to follow H_2O_2 generation and consumption rates in an EGCG-containing emulsion system and to assess the extent that proteins affect these rates. An emulsion system was used to investigate the effects of microenvironments arising from the different emulsion components (lipid, surfactant, and proteins) on EGCG oxidation and, thus, H_2O_2 generation.

MATERIALS AND METHODS

Materials. Tween 80, xylenol orange tetrasodium salt, and ferrous sulfate heptahydrate were purchased from Sigma (St. Louis, MO). Sodium caseinate (coded Alanate 191) and WPI (coded Alacen 895) were obtained from New Zealand Milk Proteins. H_2O_2 (30% w/v) was purchased from EMD Chemicals (Gibbstown, NJ), and EGCG (93% purity) was from Taiyo Green Power Co. (Jiangsu, China). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Matheson Coleman & Bell (Norwood, OH), and *n*-hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ). Guanidine hydrochloride and D-sorbitol (98% purity) were purchased from Alfa Aesar (Ward Hill, MA). Trichloroacetic acid (crystalline) was purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals and solvents were of analytical or high-performance liquid chromatography (HPLC) grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

Emulsion Preparation. Oil-in-water emulsions were prepared by dispersing 10 wt % hexadecane in 10 mM phosphate buffer (pH 7.0) with 2 wt % emulsifier (Tween 80 or caseinate). Hexadecane, a saturated hydrocarbon, was selected as a model for the lipid phase due to the fact that it is chemically stable and is not readily oxidized to lipid hydroperoxides. Sodium azide (0.02 wt %) was added to emulsions to prevent microbiological spoilage. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) on high speed for 1 min. Fine emulsions were prepared by passing coarse emulsions through a twin-stage valve homogenizer (Niro Soavi Panda, GEA Niro Soavi, Hudson, WI) for at least three passes at a pressure of 24 or 40 MPa for Tween- or caseinate-stabilized emulsions, respectively. All emulsions had a mean particle size (d_{32}) of 0.59 ± 0.02 μm, as determined by laser light scattering (Horiba LA 920, Irvine, CA). EGCG (0.5 or 2.4 mM final concentrations) was added to a 10 wt % hexadecane emulsion from a 10 mM stock solution prepared in 10 mM phosphate buffer (pH 7). The final hexadecane concentration of each emulsion was 5 wt %. Iron (25 μM ferric ions from ferric chloride, $FeCl_3$) was added to the 5 wt % hexadecane emulsion from a freshly prepared stock solution of $FeCl_3$ (10 mM) in water. Emulsions were held in the absence of light at 37 °C. Samples were analyzed over time to follow EGCG oxidation, H_2O_2 concentration, and protein carbonyl formation in caseinate-containing samples.

Hydrogen Peroxide Analysis. Emulsion samples (162.4 μL) were chemically destabilized by the addition of 2:1 methylene chloride:methanol (1 mL) followed by vortex mixing for 30 s and centrifugation at 800*g* for 2 min. Protein in caseinate-stabilized emulsions was precipitated by the addition of 0.5 N H_2SO_4 (10 μL) in addition to 2:1 methylene chloride:methanol (1 mL) followed by storage for 10 min on ice. Caseinate-containing samples were mixed for 30 s followed by centrifugation at 11000*g* for 5 min. The upper aqueous layer was collected and stored at −80 °C until analysis. A modified version of the concentrated FOX assay[36] was used for H_2O_2 analysis. The FOX assay solution consisted of xylenol orange (1 mM), ferrous sulfate (2.5 mM), and sorbitol (1.0 M) in sulfuric acid solution (0.5 N) and was prepared daily from stock solutions. Peroxide analysis was performed by

adding the assay solution (20 μL) to the aqueous extract (140 μL). Samples were diluted as necessary using a 1:2 water:methanol mixture. Samples were mixed by vortex, and absorbance values were read at 560 nm following incubation (30 min; ambient temperature) using an Agilent 8453 UV−vis diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). Quantitation was performed using an external standard curve prepared using authentic H_2O_2, the concentration of which was validated using the peroxide's extinction coefficient $\varepsilon_{240} = 40\ M^{-1}\ cm^{-1}$.

EGCG Analysis. EGCG was extracted from emulsions in the same manner as described above for H_2O_2 analysis. To prevent further EGCG oxidation during storage and analysis, a preservative solution (10 μL) consisting of ascorbic acid (20 wt %) and ethylenediaminetetraacetic acid (EDTA; 0.1 wt %) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 μL) prior to storage at −80 °C.[37] Preservative solutions were prepared daily. EGCG in extracts was measured by HPLC according to a modified method from Hu et al.[38] Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 mm × 150 mm, 5 μm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump with sample introduction by means of a 20ADvp temperature-controlled autosampler (4 °C). Samples were filtered over 0.45 μm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 25−45% B in 8.5 min. The injection volume was 20 μL, and the flow rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-M10Avp photodiode array detector, with quantitation based on an external standard curve prepared from EGCG.

Protein Carbonyl Analysis. Protein carbonyls were measured as their hydazones, which were prepared by derivatization with DNPH.[39,40] Briefly, emulsion samples (250 μL) were mixed with DNPH (10 mM; 250 μL) prepared in HCl (2 N). Samples were incubated in a water bath (37 °C; 1 h), with vortex mixing every 10 min. Following incubation, trichloroacetic acid (50% w/v; 162.4 μL) was added. Samples were placed on ice for 10 min and centrifuged for 5 min at 13000*g*. The supernatant was decanted, and the protein precipitate was washed with 1:1 ethanol:ethyl acetate (v/v; 1 mL). Samples were vortexed and centrifuged for 5 min at 11000*g*. The supernatant was redecanted, and the wash steps were repeated twice more. The resulting protein pellet was dissolved in guanidine hydrochloride (6 M; 1 mL) in sodium phosphate buffer (pH 2.3; 20 mM) and incubated at 37 °C for 15 min. Insoluble material was removed following incubation by centrifugation (2000*g*; 2 min). The absorbance values of the hydrazone derivatives were read at 375 nm. The concentration of protein carbonyls (nmol carbonyl/mg protein) was calculated based on the reported extinction coefficient for the hydrazones, $\varepsilon_{375} = 22000\ M^{-1}cm^{-1}$.

Kinetic Analysis of H_2O_2 Quenching by Proteins. The reactions between H_2O_2 and the proteins sodium caseinate and WPI were carried out with the proteins present in either phosphate buffer alone, as nonadsorbed aqueous phase solutes in Tween-stabilized emulsions, or at emulsion interfaces (i.e., the proteins were used as emulsifiers in model emulsions). Surfactant-stabilized 10 wt % hexadecane O/W emulsions were prepared with Tween 80 (2 wt %) in 10 mM phosphate buffer (pH 7.0). Protein-stabilized emulsions were prepared with 5 wt % hexadecane and the specified protein concentration (0.5−2.0 wt %) in phosphate buffer (pH 7.0; 10 mM). The mean particle sizes (d_{32}) of caseinate-, WPI-, and Tween-stabilized emulsions were 0.52 ± 0.04, 0.50 ± 0.04, and 0.47 μm, respectively. For samples with protein in phosphate buffer alone, the specified protein concentrations were achieved by diluting 10 wt % sodium caseinate or WPI solutions in 10 mM phosphate buffer to the desired final concentrations (0−2 wt % protein). For Tween-stabilized systems, emulsions were diluted with the protein stock solutions and buffer to prepare 5.0 wt % hexadecane emulsions with the desired protein concentrations ranging from 0 to 2 wt % protein. Protein-stabilized emulsions were used as is. Sodium azide (0.02 wt %) was added to all samples. Reactions were initiated by the addition of H_2O_2 (2.0 mM). Samples were mixed by vortex and stored in the absence of light in a 25 °C shaking water bath with periodic sampling for H_2O_2 analysis.

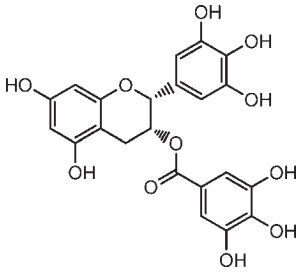

Figure 1. Structure of EGCG.

Statistical Analysis. All experiments were performed in triplicate, and results were expressed as means and standard deviations. Two-way analysis of variance with Bonferroni's post-test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Hydrogen Peroxide Generation in Surfactant- and Protein-Stabilized Emulsions. EGCG concentrations were followed by HPLC, with nearly all EGCG lost in both surfactant- and protein-stabilized systems within 96 h of storage at pH 7 (Figure 2a). EGCG showed significantly lower oxidative stability in the presence of protein, suggesting that it may also generate H_2O_2 at a faster rate, as catechol oxidation and oxygen reduction reactions appear to be linked.[41,42] While few studies have directly examined the oxidative stability of phenolic compounds in protein-containing foods, some have studied this chemistry under physiological conditions. For example, human serum albumin was observed to increase EGCG stability in buffer at neutral and alkaline pH.[43,44] The EGCG concentration was also shown to be more stable in the presence of *N*-acetyl cysteine and reduced glutathione (GSH), while the opposite was observed in the presence of oxidized glutathione (GSSG).[45] Under wine conditions (pH 3.6), the presence of nucleophiles such as benzenesulfinic acid was seen to markedly increase 4-methylcatechol oxidation, as measured by dissolved oxygen consumption.[46] The authors argued that the benzenesulfinic acid accelerated oxidation of the catechol by reacting rapidly with its oxidation product, the benzoquinone, by a 1,4-Michael type addition reaction, thus shifting the equilibrium to the right. Therefore, the fact that EGCG oxidized faster in the presence of caseinate in our system may be attributable to the quenching of EGCG quinones by nucleophilic thiol groups (e.g., cysteine residues) on the protein. EGCG quinones are known to form covalent adducts with thiol residues,[47,48] although lysine and tryptophan adducts have also been reported.[49]

An alternative explanation for the observed differences in EGCG oxidation between surfactant- and protein-stabilized systems may be due to the multiphasic nature of the system that allows EGCG to partition into the lipid phase or surfactant micelles. Tea catechins displayed greater oxidative stability in the presence of Tween 20 or lecithin, because of partitioning into the amphiphilic structures, reducing the likelihood of interacting with

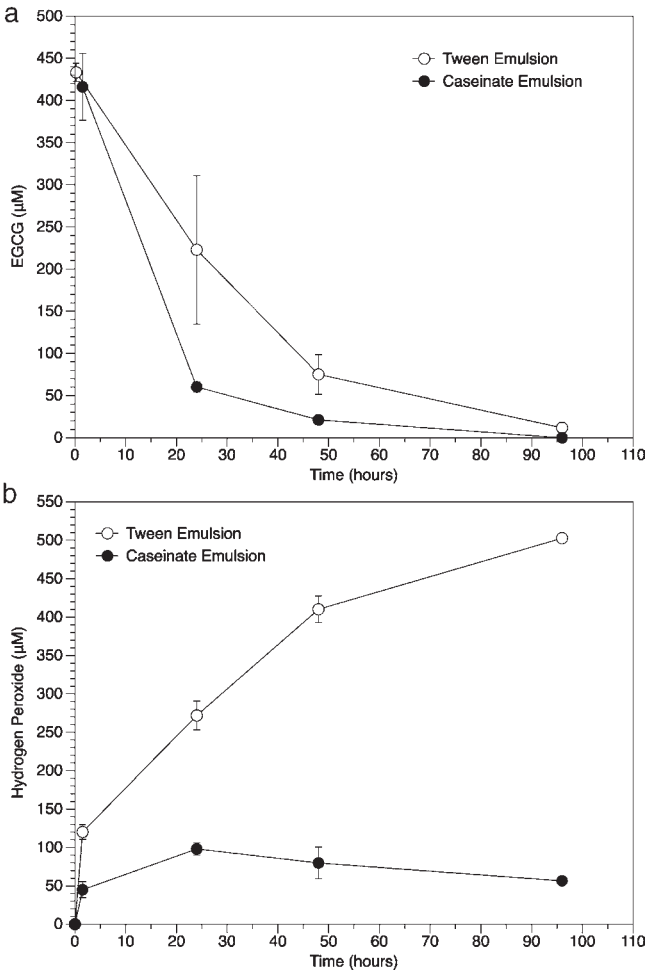

Figure 2. Changes in (a) EGCG concentration and (b) H_2O_2 concentration resulting from the oxidation of 0.5 mM EGCG in Tween- and caseinate-stabilized 5 wt % hexadecane emulsion in 10 mM phosphate buffer (pH 7).

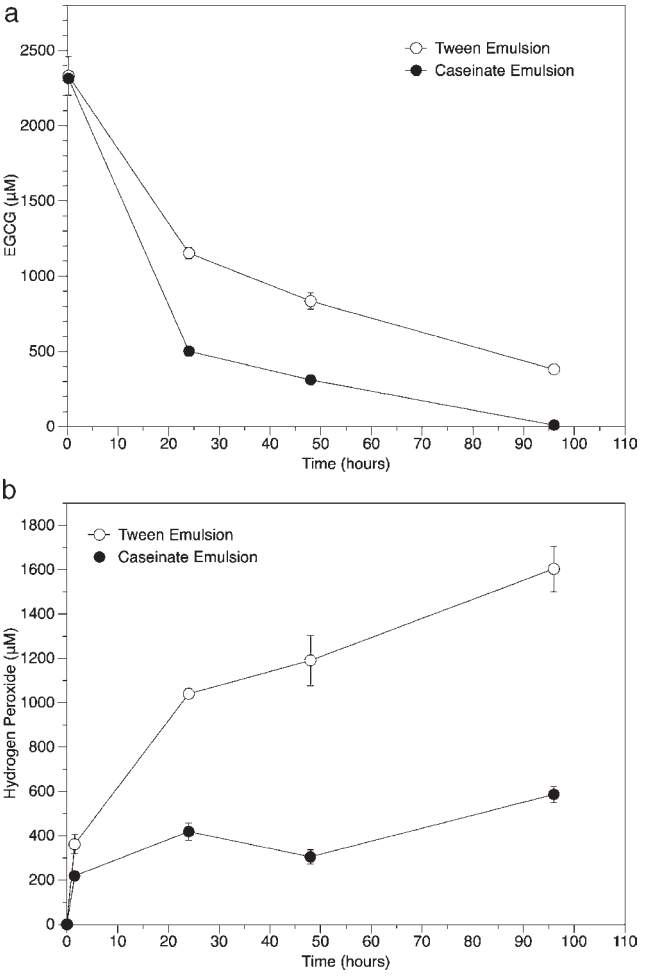

Figure 3. Changes in (a) EGCG concentration and (b) H_2O_2 concentration resulting from the oxidation of 2.4 mM EGCG in Tween- and caseinate-stabilized 5 wt % hexadecane emulsion in 10 mM phosphate buffer (pH 7).

radicals or transition metals that may otherwise promote its oxidation.[50]

The generation of H_2O_2 due to EGCG (starting concentration, 0.5 mM) oxidation in the presence of iron (25 μM Fe^{3+}) in surfactant and protein-stabilized emulsions was examined over 96 h (Figure 2b). As the FOX assay is not specific to H_2O_2, catalase was added at the end of each study to determine if other peroxides were formed. No other peroxide species (e.g., hydroperoxides) were observed (data not shown). Samples in Tween-stabilized emulsions showed no further increase after day 4 as levels plateaued at ca. 500 μM H_2O_2 and persisted throughout the 8 day study (data not shown). At all time points, samples in protein-stabilized emulsions showed significantly lower levels of H_2O_2 as compared to the surfactant-stabilized system. A similar trend for EGCG oxidation and H_2O_2 generation was observed in the presence of 2.4 mM EGCG. In Tween-stabilized emulsions, ca. 52% of the EGCG (corresponding to ~1.25 mM) was oxidized, while in protein-stabilized emulsions, ca. 79% of the EGCG (corresponding to ~1.9 mM) was oxidized within the first 24 h (Figure 3a), suggesting that most of the H_2O_2 was generated during this time. The H_2O_2 concentration continuously increased in Tween-stabilized emulsions to a final concentration of 1.6 mM at the end of 96 h (Figure 3b). However, protein-stabilized emulsions displayed H_2O_2 concentration well below Tween-stabilized emulsions throughout the study.

The difference in observed H_2O_2 levels in surfactant- and protein-stabilized emulsions is likely due to the peroxide scavenging activity of the protein. It is unlikely that H_2O_2 generation rates were slower in the protein-containing system given the relative instability of EGCG in that same system, as oxygen reduction rates, and thus H_2O_2 formation rates, should mirror EGCG oxidation rates. Further evidence of H_2O_2 scavenging by protein can be seen by measuring protein oxidation. Therefore, protein carbonyl concentration, a general marker of protein oxidation, was measured in protein-stabilized emulsions in the presence of 0.5 and 2.4 mM EGCG (Figure 4). Protein carbonyl concentrations were observed to increase with increasing EGCG concentrations, most likely as a result of the protein reacting with H_2O_2. In protein-stabilized emulsions containing 0.5 mM EGCG, protein carbonyl concentrations increased markedly within 24 h, coinciding with a large reduction in H_2O_2 concentration during that time (as seen in Figure 2b), even as ca. 89% of the catechin (corresponding to ~0.44 mM EGCG) had been oxidized. When 2.4 mM EGCG was added, the protein carbonyl content once again showed a sharp increase in the first 24 h, as 79% of the

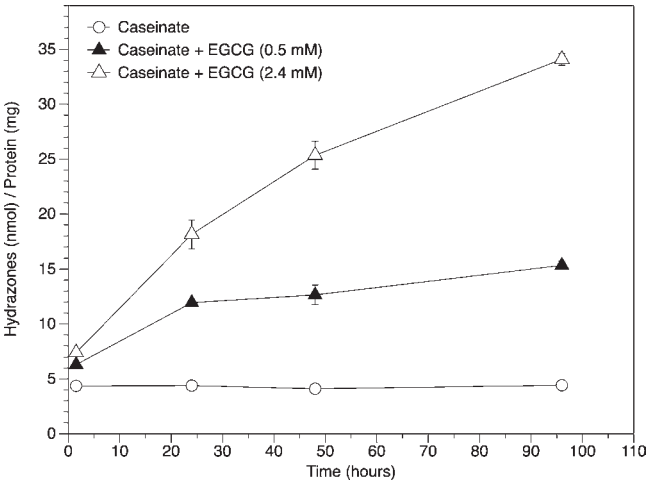

Figure 4. Protein carbonyl formation resulting from EGCG oxidation in caseinate-stabilized 5 wt % hexadecane emulsion.

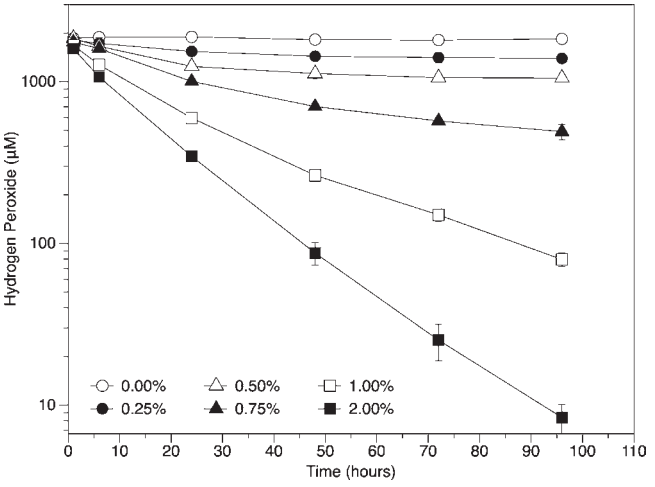

Figure 5. Quenching of H_2O_2 by caseinate solutions (0−2.0 wt %) in 10 mM phosphate buffer pH 7.0.

catechin oxidized to produce H_2O_2, with H_2O_2 concentrations significantly (~600 μM) below concentrations observed in Tween-stabilized emulsions. EGCG may also covalently bind to proteins to form adducts that may retain their redox activity[51] and further induce oxidative stress on the protein by generating more H_2O_2 near the adduct site. Thus, H_2O_2 was added exogenously to proteins in the latter quenching experiments.

Hydrogen Peroxide Quenching by Aqueous Phase and Interfacial Proteins in Emulsions. The H_2O_2 scavenging activity of sodium caseinate and WPI as aqueous solutes and as components of oil-in-water emulsions was examined in the following experiment. The concentration-dependent quenching of exogenous H_2O_2 (2 mM starting concentration) by caseinate solutions in 10 mM phosphate buffer pH 7 was measured over 96 h (Figure 5). The quenching of H_2O_2 by caseinate appears to follow first order reaction kinetics at high protein concentrations (1.0 and 2.0 wt %). The highest concentration of caseinate (2.0 wt %) tested quenched nearly all of the added peroxide (2 mM H_2O_2). This suggests that the concentration of caseinate (1 wt %) used in the previous protein-stabilized emulsion was incapable of quenching all of the H_2O_2 generated from the oxidation of 2.0 mM EGCG.

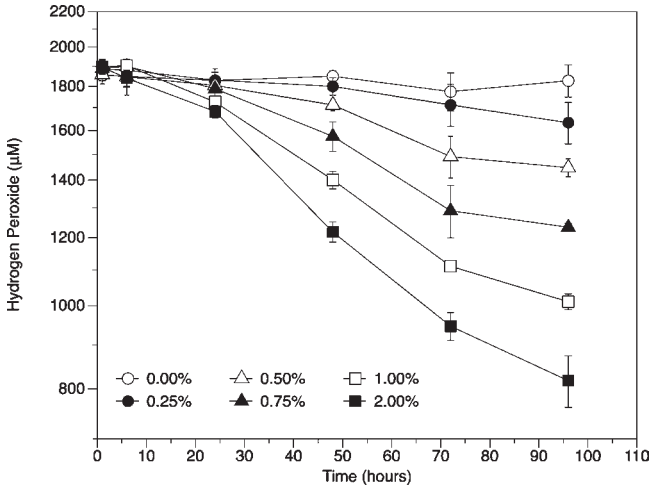

Figure 6. Quenching of H_2O_2 by WPI solutions (0−2.0 wt %) in 10 mM phosphate buffer pH 7.0.

The peroxide scavenging activity of WPI in 10 mM phosphate buffer pH 7 was also measured for 96 h (Figure 6). Unlike caseinate solutions, WPI solutions did not show first order reaction kinetics, even at higher protein concentrations. WPI solutions displayed a significantly lower capacity for scavenging peroxides, as 2.0 wt % caseinate was capable of scavenging virtually all of the added H_2O_2, while the equivalent concentration of WPI was only capable of scavenging ca. 61% of the peroxide (1.2 mM H_2O_2). This difference in protein scavenging activity may be a function of the protein's primary amino acid sequence as well as the accessibility of certain amino acids to the aqueous solvent phase. Amino acids that are known to react with peroxides include cysteine[52,53] and methionine.[54] α_{s2}-Casein and κ-casein both contain two cysteine residues, whereas α_{s1}-casein and β-casein lack free cysteines. The predominant whey proteins, β-lactoglobulin and α-lactalbumin contain five and eight cysteine residues, respectively. Although it appears that the proteins in WPI contain more cysteines, many of these residues are not solvent accessible[55] or participate in disulfide linkages.[56,57] β-Lactoglobulin contains two disulfide linkages,[58] while α-lactalbumin may be stabilized by four disulfide bonds,[59,60] thus limiting the cysteine's reactivity. With respect to solvent accessibility, it has been shown that enzymatic hydrolysates have increased antioxidant activity in lipid systems,[61] presumably due to increased solvent accessibility of radical scavenging amino acids.[34] Methionine residues have also been shown to reduce peroxide species via a two-electron (nonradical) reaction.[18,19] Levine et al. showed that the H_2O_2-induced oxidation of bacterial glutamine synthetase caused an increase in methionine sulfoxide formation. It was observed that the most oxidatively labile methionine residues were those that were solvent exposed, while those residues buried within the protein's hydrophobic core remained unaffected.[62] Methionine residues have been studied previously for their antioxidant activity, and many studies have also shown that this activity is a function of the solvent accessibility to key amino acid residues.[63−65]

On the basis of solvent accessibility calculations, three of β-lactoglobulin's methionine residues were determined to be buried within the protein's hydrophobic core and, thus, not solvent accessible. Methionine residues in β-lactoglobulin in that study were shown to be oxidatively stable in a surfactant-stabilized

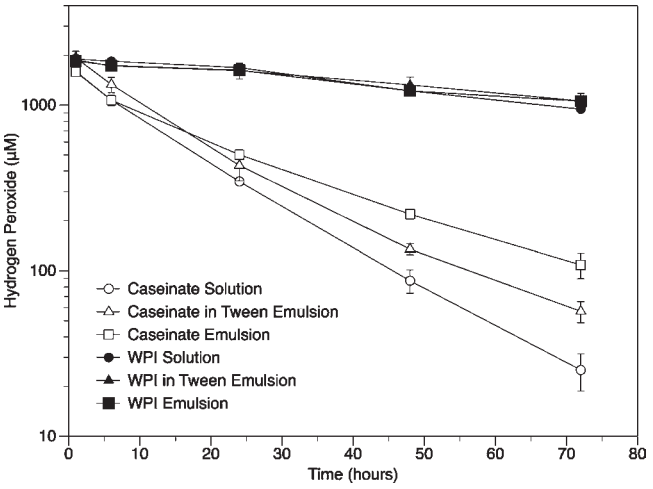

Figure 7. Quenching of H_2O_2 by 1.0 wt % caseinate or WPI in buffer only, Tween-stabilized emulsion, and protein-stabilized emulsion.

emulsion containing the protein as an aqueous phase solute. Furthermore, on the basis of our calculations using the GETAREA algorithm,[66] α-lactalbumin's sole methionine residue does not appear to be solvent accessible either. Crystallography data for the casein fractions comprising sodium caseinate were not available at the time of writing; therefore, solvent accessibility data for its methionine residues could not be calculated. However, it has been shown that ca. 75% of bovine casein's methionine residues were capable of undergoing oxidation to their sulfoxides under similar conditions (6 mM H_2O_2) to those employed in the above-described experiment.[67]

The peroxide scavenging activity of caseinate and WPI were also investigated in various systems, which included the proteins as solutes in a simple buffer system, as aqueous phase components of surfactant-stabilized emulsions and as emulsifiers themselves. The peroxide scavenging activity in the various protein (1.0 wt %) containing systems was examined for 72 h (Figure 7). No significant differences ($p < 0.05$) between WPI treatments (WPI solution vs WPI in Tween emulsion vs WPI emulsion) were observed over the course of the study; however, a marked difference between caseinate treatments was observed. The rate of H_2O_2 consumption by caseinate solutions exceeded that of caseinate in both surfactant- and protein-stabilized systems. The rate of H_2O_2 was slowest in the caseinate-stabilized emulsion system. We hypothesize that this effect may be due to the adsorption behavior of casein. In one study, it was shown that β- casein preferentially adsorbed to the droplet interfaces of 30% soybean oil-in-water emulsions stabilized with <2.0 wt % casein, while at higher protein concentrations, α-casein was preferentially adsorbed.[68] Our study employed lower total oil levels, so it may be possible that in the case of a 5 wt % hexadecane emulsion, α-casein may be preferred to stabilize the emulsion droplet. The predominance of α-casein at the emulsion interface may reduce the ability of the protein to scavenge H_2O_2, as α-casein will no longer be free to interact with the peroxide in the aqueous phase. This is consistent with the observation that aqueous phase α-casein has a higher radical scavenging activity[69] and antioxidant properties by delaying lipid peroxidation induction time in liposomal suspensions[70] as compared to the other casein proteins.

Proteins appear to play a significant role in the production and fate of H_2O_2 in polyphenol-containing foods. While sodium caseinate appeared to accelerate EGCG oxidation and, in turn, H_2O_2 production rates, the protein was observed to rapidly quench the peroxide in a concentration-dependent manner, preventing its accumulation. H_2O_2 quenching rates were seen to vary between caseinate and WPI, with higher peroxide scavenging observed with the aqueous phase caseinate. Further research is needed to elucidate which amino acid residues are responsible for H_2O_2 quenching under these conditions and how this quenching translates to lipid oxidation inhibition in a real food or beverage system.

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