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Antioxidant Activity of Cysteine, Tryptophan, and Methionine Residues in Continuous Phase β-Lactoglobulin in Oil-in-Water Emulsions

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Proteins dispersed in the continuous phase of oil-in-water emulsions are capable of inhibiting lipid oxidation reactions. The antioxidant activity of these proteins is thought to encompass both free radical scavenging by amino acid residues and chelation of prooxidative transition metals; however, the precise mechanism by which this occurs remains unclear. In this study, the oxidative stability of cysteine, tryptophan, and methionine residues in continuous phase β-lactoglobulin (β-Lg) in a Brij-stabilized menhaden oil-in-water emulsion was determined. The presence of low concentrations of continuous phase β-Lg (250 and 750 μg/mL) significantly inhibited lipid oxidation as determined by lipid hydroperoxides and thiobarbituric acid reactive substances analysis. It was observed that cysteine oxidized before tryptophan in β-Lg, and both residues oxidized before lipid oxidation could be detected. No oxidation of the methionine residues of β-Lg was observed despite its reported high oxidative susceptibility. It is conceivable that surface exposure of amino acid residues greatly affects their oxidation kinetics, which may explain why some residues are preferentially oxidized relative to others. Further elucidation of the mechanisms governing free radical scavenging of amino acids could lead to more effective applications of proteins as antioxidants within oil-in-water food emulsions.

KEYWORDS: Amino acids; antioxidants; β-lactoglobulin; lipid oxidation; protein oxidation; emulsions

INTRODUCTION

Lipid oxidation is one the major causes of food spoilage, resulting in off flavors and off odors (*1*, *2*). Long-chain ω-3 fatty acids, such as those commonly found in marine oils, have been shown to deliver significant health benefits but are extremely labile with regard to their oxidative stability (*3*). It is, therefore, necessary to protect these oils to maintain their health benefits and to ensure consumer acceptability. Because of the fact that most dietary lipids are not consumed in their bulk state, fats and oils in processed foods are typically encountered as emulsions (*4*). Proteins have been shown to inhibit lipid oxidation both at the interface of emulsion droplets and within the continuous phase of oil-in-water emulsions (*5*–*7*). The former mechanism is thought to involve both free radical scavenging by certain amino acid residues and electrostatic repulsion of cationic prooxidants, such as transition metals, at pH values below the interfacial protein's isoelectric point. The overall antioxidant mechanism of proteins most likely encompasses free radical scavenging of aqueous phase or surface active oxidants and chelation of transition metals. However, the precise mechanism by which proteins act as antioxidants in oil-in-water emulsions is unclear.

Milk proteins, such as β-casein and β-lactoglobulin (β-Lg), are often utilized as emulsifying agents in foods and have been shown to undergo oxidative modification at the interface of emulsion droplets (*8*, *9*). The most common example of protein modification in milk protein-stabilized oil-in-water emulsions occurs by Michael addition reactions (*9*–*11*); however, lipid-derived oxidation products can lead to oxidation of amino acid residue side chains (*12*). For a given amino acid residue to be an antioxidant, it must oxidize preferentially to polyunsaturated fatty acids. Amino acid residues vary greatly with regard to their individual oxidative stability (*13*); therefore, the ability of these residues to interact with lipid-derived free radicals and hydroperoxides affects the protein's overall antioxidant activity at an emulsion interface (*9*). For example, the pseudo-first-order rate constants of amino acid side chains oxidized with hydroxyl radical have been measured, and their relative reactivity rate was observed to be Cys > Trp, Tyr > Met > Phe > His > Ile > Leu > Pro (*14*). Therefore, proteins with high effective concentrations of oxidatively labile amino acid residues (e.g., Cys, Trp, and Met) have the potential to inhibit lipid oxidation reactions.

Proteins dispersed in the continuous phase of emulsions are susceptible to oxidative modification and, in some cases, can act as antioxidants. Faraji et al. reported that continuous phase whey protein plays a major role in protecting oxidatively labile emulsified lipids (*15*). Of the whey proteins, β-Lg acts as an antioxidant but is slightly less effective at inhibiting lipid oxidation than other whey components, such as α-lactalbumin (α-La) (*8*). Allen et al. attributed the antioxidant effect of β-Lg

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to the fact that it contains a free sulfhydryl group (Cys121) that is partially buried within the protein core (*8*). However, it was unexplained as to why α-La had a stronger antioxidant effect, given that it contains no free sulfhydryl groups (*6*, *8*). Cysteine is thought to act as an antioxidant by donating a hydrogen from its thiol group (*16*, *17*). Despite the relative inaccessibility of this residue, β-Lg's lone free sulfhydryl group does play a role in inhibiting lipid oxidation by scavenging free radicals (*6*). However, it has been reported that cysteine is not the only amino acid residue responsible for free radical scavenging (*18*); therefore, a study designed to assess which amino acid residues are capable of inhibiting lipid oxidation is necessary.

Levine et al. propose that methionine, a sulfur-containing amino acid whose precise biological role remains unknown, constitutes an important endogenous antioxidant defense mechanism in proteins (*18*). Surface-exposed methionine in bacterial glutamine synthetase is readily oxidized to methionine sulfoxide in the presence of millimolar concentrations of hydrogen peroxide (*18*). Levine et al. also report that up to six methionine residues per subunit of glutamine synthetase can be oxidized before any appreciable effect on the protein structure is observed (*18*). Methionine sulfoxide reductases, capable of reducing either free or protein-bound methionine sulfoxide back to methionine, are widespread among many organisms (*19*), indicating that methionine oxidation is reversible and relatively commonplace. As endogenous components of protein, the concentration of methionine residues is very high, providing effective scavenging of oxidants before they can attack components that are critical to cellular structure or function (*18*).

Hernández-Ledesma et al. investigated the radical scavenging activity of several amino acid and peptide fragments obtained via enzymatic hydrolysis of β-Lg and α-La by calculating their oxygen radical absorbance capacity (ORAC) values (*16*). Tryptophan was determined to have the highest antioxidant activity, followed by methionine and cysteine, respectively. Several peptides were found to possess radical scavenging capacity, including the sequence Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile, which had a higher ORAC value than the synthetic, food-grade commercial antioxidant, butylated hydroxyanisole (*16*). Tryptophan is thought to act as an antioxidant by serving as a hydrogen (H^+) donor, thus allowing oxygen radicals to quench its indolic hydrogen (*16*).

The primary objective of this study was to determine the antioxidant capacity of three oxidatively labile amino acid residues (free cysteine, methionine, and tryptophan) in β-Lg dispersed in the continuous phase of a fish oil-in-water emulsion. The effect of continuous phase surfactant and the antimicrobial agent thimerosal on the detection of free sulfhydryl groups was also assessed. The ultimate goal of this study is to elucidate the mechanisms by which continuous phase β-Lg acts as an antioxidant in order to design an oxidatively stable emulsion delivery system of lipids high in bioactive polyunsaturated fatty acids.

MATERIALS AND METHODS

Materials. Food-grade lyophilized β-Lg was donated by Davisco Foods International, Inc. (Eden Prairie, MN). Unstabilized, deodorized, refined, and bleached menhaden oil (eicosapentaenoic acid, 10−17%; docosahexenoic acid, 7−12%) was donated by Omega Protein (Houston, TX). 7-Fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) was obtained from Molecular Probes, Inc. (Eugene, OR). Polyoxyethylene (20) sorbitan monolaurate (Tween 20) and polyoxyethylene (20) sorbitan monooleate (Tween 80) were donated by Uniqema (Wilmington, DE). All other chemicals were of reagent or high-performance liquid chromatography (HPLC) grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Methods. *Preparation of Emulsion.* Brij 35-stabilized menhaden oil-in-water emulsions were used in all lipid oxidation studies. The emulsifier solution was prepared by dispersing Brij 35 in phosphate-buffered saline (PBS; 10 mM phosphate/0.15 M NaCl, pH 7.0) and stirring for 1 h at ambient temperature. Menhaden oil was added to the aqueous emulsifier solution and homogenized at high speed for 2 min with a hand-held biohomogenizer (Biospec Products Inc., Bartlesville, OK) to produce a 20% (w/w) oil-in-water coarse emulsion. The emulsion droplet size was then further reduced with an ultrasonicator (Fisher Sonic Dismembrator 500) at 4 °C for 3 min at 70% power and 0.5 duty cycle. A laser light scattering instrument (Coulter LS-230; Miami, FL) was used to measure the particle size distribution. The mean particle size of the secondary emulsion ranged from 0.68 to 0.80 μm and remained unchanged throughout the course of the study.

Removal of Continuous Phase Surfactant. The stock emulsion (∼45 mL aliquots) was divided among three centrifuge tubes (Sorvall 75 mL, 35 mm × 80 mm; Asheville, NC) and centrifuged at 36000*g* at 4 °C for 60 min. After separation, the continuous phase (lower layer) was removed by syringe and discarded, and an equal volume of fresh PBS was added. The emulsion was dispersed by vortexing for 2 min. This procedure of centrifugation, removal of the continuous phase, and reconstitution of the emulsion droplets was repeated a total of three times. Following the final wash, the total lipid content of the emulsion was determined using a modification of Bligh and Dyer's method (*20*). The emulsion was then adjusted to a lipid concentration of 5% (w/w) by diluting with β-Lg in fresh buffer (final protein concentration of 250 or 750 μg/mL of emulsion) containing 0.02% sodium azide as an antimicrobial agent. The control emulsion was diluted with buffer containing only sodium azide.

Lipid Oxidation Measurements. Emulsions (3 mL) were placed in capped test tubes (Fisherbrand 13 mm × 100 mm) and allowed to autooxidize in the absence of light at 20 °C for up to 10 days. Lipid hydroperoxides were measured according to Shantha and Decker's method by mixing the emulsion (0.3 mL) with 1.5 mL of isooctane/1-butanol (2:1, v/v), followed by 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$) (*21*). After 20 min, the absorbance of the solutions was measured at 510 nm using an Amersham Pharmacia Biotech Ultrospec 3000 Pro UV−vis scanning spectrophotometer (Cambridge, England). Hydroperoxide concentrations were determined using a standard curve prepared with cumene hydroperoxide.

Thiobarbituric acid reactive substances (TBARS) (*22*) were determined by mixing between 0.1 and 1.0 mL (final volume adjusted to 1.0 mL with double-distilled water) of emulsion with 2.0 mL of TBA reagent (15% w/v trichloloracetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then centrifuged (1000*g*) for 15 min. After 10 min, the absorbance was measured at 532 nm. Concentrations of TBARS were determined using a standard curve prepared using 1,1,3,3-tetraethoxypropane.

Quantification of Amino Acid Modification. All amino acid oxidation assays were performed on continuous phase protein that was free of Brij-stabilized oil droplets. Emulsions were centrifuged (1000*g*) for 20 min in Millipore Amicon Ultra centrifuge membrane filters (15 mm × 115 mm, 100000 MWCO; Billerica, MA), which retained the emulsion droplets and yielded a clear filtrate of β-Lg in buffer. The protein concentration was measured according to the Lowry method (*23*). All filtered β-Lg solutions used for methionine analysis were stored at −80 °C. Tryptophan residues were measured directly by fluorescence (excitation = 280 nm, emission = 331 nm) on a Hitachi F-2000 fluorometer (Tokyo, Japan) following dilution in PBS (*24*). Free cysteine residues were derivatized with ABD-F and measured directly by fluorescence (excitation = 365 nm, emission = 492 nm) following dilution by phosphate buffer (100 mM phosphate buffer, pH 8.0, and 1 mM diethylenetriaminepentaacetic acid) (*25*). The ABD-F thiol probe was added (10 μL of 10 mM ABD-F in PBS) to the diluted β-Lg solutions, mixed by vortex, and incubated in a 60 °C water bath for 20 min.

Methionine oxidation was assessed in β-Lg samples by treatment with CNBr followed by acid hydrolysis and separation by HPLC (*18*,

26). Protein samples (75 μg) were lyophilized under nitrogen gas in 1 mL reaction vials (Alltech 10 mm × 40 mm; Deerfield, IL) fitted with PTFE-lined caps. A stock solution of CNBr (10 M CNBr in acetonitrile) was diluted immediately before use to 100 mM with 70% v/v formic acid, of which 100 μL was added to the dried protein (*18*). CNBr cleaves peptide bonds on the carboxyl side of methionine, yielding homoserine, but does not cleave at methionine sulfoxide (*18*). The samples were incubated (Multi-Blok 2053, Lab-Line Instruments, Inc; Melrose Park, IL) at 70 °C for 1 h, cooled for 30 min at room temperature, and transferred to hydrolysis tubes (Pierce 29550, 1 mL, 8 mm × 60 mm; Rockford, IL). Following lyophilization under nitrogen gas, the CNBr-treated samples were hydrolyzed in 6 N HCl at 155 °C (Pierce Reacti-Therm 18900 Heating/Stirring Module; Pierce Chemical Co.) for 30 min in the presence of 2 mM DL-1,4-dithiothreitol (DTT), 0.2% phenol, and 0.2% benzoic acid (*26*). The hydrolysates were dried under nitrogen gas, dissolved in 100 μL of HPLC grade deionized water, and kept at −80 °C until use.

The CNBr-treated hydrolysates were separated by reverse phase HPLC (Shimadzu LC-10AT; Kyoto, Japan) equipped with a C18 column (Vydac Denali 238DE5415; Grace Vydac, Hesperia, CA) following *o*-phthalaldehyde (OPA) derivatization. The hydrolysates were eluted with a gradient beginning with 90% buffer A (35 mM sodium acetate containing 4% v/v HPLC grade tetrahydrofuran, pH 5.7) and 10% solvent B (HPLC grade methanol). Solvent B was linearly increased to 65% over 20 min and returned to 10% in 5 min. The flow rate was maintained at 1.5 mL/min. The OPA-derivatized hydrolysates were detected by fluorescence (excitation = 325 nm, emission = 465 nm) on an online Waters 474 scanning fluorescence detector (Millipore Corp.). Methionine was detected as homoserine and compared to oxidatively stable endogenous amino acid residues present in β-Lg.

Assessing the Effect of Dispersed Surfactants and Thimerosal on Cysteine Measurement. The effect of continuous phase surfactants (Brij 35, Tween 20, and Tween 80) on the accessibility of cysteine residues to the ABD-F probe was assessed by adding 10 or 100 μL of 17 mM surfactant solution to 100 μg β-Lg/mL phosphate buffer or 8 M urea (final volume 1 mL). In some cases, surfactant solutions were either added to protein samples prior to denaturation with 8 M urea or directly to denatured protein samples. The effect of continuous phase thimerosal on cysteine detection was assessed by adding thimerosal (1 mM) to the continuous phase of the washed emulsion (5% w/w lipid). The continuous phase of emulsion samples was separated from the dispersed phase by centrifuge membrane filters described previously. Free cysteine residues were derivatized with ABD-F as described previously following dilution by phosphate buffer or 8 M urea.

Statistical Analysis. All experiments were performed on triplicate samples. Statistical analysis was performed using Student's *t*-test (*27*).

RESULTS AND DISCUSSION

Effect of Thimerosal on Cysteine Detection. The antimicrobial agent thimerosal (merthiolate) is commonly used to inhibit microbial growth in emulsion systems undergoing shelf life testing. Thimerosal is an organic mercury compound, consisting of an organic radical, ethylmercury, which is bound to the sulfur atom of the thiol group of salicylic acid (*28*). The presence of thimerosal in the continuous phase of oil-in-water emulsions is an effective antibacterial and antifungal agent; however, it was observed that this compound interferes with cysteine analysis. The fluorometric intensity of 100 μg β-Lg/mL PBS derivatized with ABD-F was reduced by 99% in the presence of thimerosal (final concentration 1 mM) as compared with the control (**Figure 1**). Total sulfhydryls in β-Lg can be determined by denaturing the protein with urea to expose buried cysteine residues. Thimerosal (1 mM) reduced 94% of the fluorometric intensity of ABD-F derivatized denatured β-Lg as compared with the control. Because denatured β-Lg treated with ABD-F did not have higher fluorescence values than native β-Lg, it appears that a reduction in fluorescence in thimerosal-containing samples is not due to a reversible, conformational change in the protein.

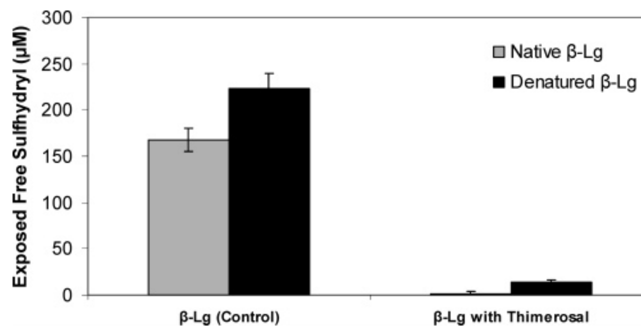

Figure 1. Influence of 1 mM (final concentration) thimerosal on the fluorescence (excitation = 365 nm, emission = 492 nm) of native β-Lg in phosphate buffer (pH 7.0) or β-Lg denatured with 8 M urea, following derivatization with ABD-F. Data points represent means ($n = 3$) ± standard deviations.

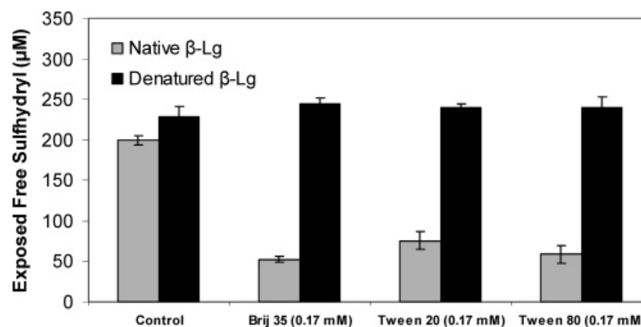

Figure 2. Effect of 0.17 mM (final concentration) Brij 35, Tween 20, and Tween 80 on the detection of β-Lg's sulfhydryl group with ABD-F. Native β-Lg in phosphate buffer (pH 7.0) was compared with β-Lg denatured with 8 M urea for 1 h. Surfactants were added to protein samples before denaturation. Data points represent means ($n = 3$) ± standard deviations.

These data suggest that the thiol groups of 1 mM thimerosal, of which there is a molar excess as compared with the theoretical maximum number of free cysteine residues present in 100 μg of β-Lg, reacts preferentially with the ABD-F probe to yield a product that does not fluoresce at the wavelengths used for cysteine detection (excitation = 365 nm, emission = 492 nm). It is also possible that thimerosal reacts preferentially with β-Lg's free sulfhydryl groups vs the ABD-F probe. Thimerosal's thiol group may also scavenge free radicals via a mechanism similar to that of cysteine; this may result in artificially lower lipid oxidation values in protein-dispersed oil-in-water emulsions. Thimerosal is thus a poor choice as an antimicrobial agent in this system. Sodium azide (NaN_3) has been established as an effective antimicrobial agent (*29*) and was observed not to interfere with cysteine detection using thiol reactive probes such as ABD-F (data not shown); therefore, sodium azide was selected for use in this study.

Effect of Excess Continuous Phase Surfactant on Cysteine Detection. A survey of the effect of three aqueous phase-dispersed surfactants on the ability of the ABD-F thiol probe to detect cysteine in β-Lg was carried out. A significant decrease ($p \leq 0.05$) in fluorescence was observed for β-Lg samples containing added surfactants (final concentration 0.17 mM) (**Figure 2**). The presence of 0.17 mM Brij 35, Tween 20, and Tween 80 resulted in a 68, 57, and 65% reduction in fluorometric intensity relative to the nonsurfactant-containing control. Polyether surfactants, such as Brijs and Tweens, commonly contain hydroperoxides that can accumulate during storage (*30*). It is possible that cysteine could be oxidized by Brij or Tween hydroperoxides, thus decreasing cysteine concentrations. However, it is also possible that the presence of surfactants, such as

Brij and Tweens, hinders cysteine quantification by the thiol probe by inducing a conformational change upon β-Lg. A change in the tertiary structure of β-Lg might reduce the accessibility of the ABD-F probe to the protein's lone free sulfhydryl group.

To determine if surfactant hydroperoxides could oxidize cysteine, 10 μL aliquots of 17 mM Brij 35, Tween 20, or Tween 80 (final concentration 0.17 mM) were added to β-Lg that had been denatured by 8 M urea. Denatured β-Lg that was treated with surfactant yielded fluorometric intensities that were not significantly different ($p \leq 0.05$) from those obtained from the denatured control (no added surfactant). Furthermore, a 10-fold increase in surfactant concentration (final concentration 1.7 mM) failed to yield fluorescence values below that of protein samples containing 0.17 mM surfactant (data not shown). These data suggest that surfactants, such as Brij and Tween, do not decrease cysteine quantification by hydroperoxide-driven oxidation reactions; therefore, surfactants could be conferring a structural change on β-Lg that either reduces the efficacy by which the ABD-F probe accesses cysteine's free sulfhydryl group or causes the cysteine−ABD-F fluorescent product to be further buried within the protein core, thus making it unable to interact with the fluorometer's light source. Given that excess surfactant was observed to affect β-Lg's conformation properties and, in turn, cysteine analysis, it was necessary to apply an extensive washing method to the emulsion in order to minimize the presence of continuous phase Brij. Faraji et al. reported that consecutive washing steps significantly reduced the concentration of continuous phase solutes (*15*). It was also important that the stock emulsion be prepared with just enough surfactant to produce a stable emulsion in order to minimize the amount of excess surfactant present in the continuous phase.

Inhibition of Lipid Oxidation by Continuous Phase β-Lg. Lipid oxidation analysis was performed on samples containing either 250 or 750 μg continuous phase β-Lg/mL emulsion (plus nonprotein containing control) over the course of 10 days. The presence of continuous phase β-Lg was observed to be effective at inhibiting lipid oxidation over the course of the study, as measured by lipid hydroperoxides and TBARS (**Figure 3A**,**B**, respectively). After 1 day of incubation, 250 and 750 μg β-Lg/mL significantly ($p \leq 0.05$) inhibited the formation of lipid hydroperoxides by 36 and 40%, respectively, vs the nonprotein containing control. By day 3 of the study, the presence of 750 μg β-Lg/mL was significantly ($p \leq 0.05$) more effective than 250 μg β-Lg/mL in inhibiting the formation of lipid hydroperoxides. Samples containing 250 and 750 μg β-Lg/mL were observed to inhibit the formation of lipid hydroperoxides by 67 and 93%, respectively, after 4 days of incubation as compared with the control. Lipid hydroperoxides remained at a low level throughout the course of the study in emulsions containing 750 μg β-Lg/mL. Emulsions containing 750 μg β-Lg/mL were observed to be significantly ($p \leq 0.05$) more effective than those containing 250 μg β-Lg/mL at inhibiting TBARS formation after 1 day. The presence of 250 and 750 μg β-Lg/mL led to a 46 and 77% inhibition of TBARS formation, respectively, vs the nonprotein containing control after 4 days of storage. To have maximum protein concentrations to be able to measure amino acid oxidation (especially with the ABD-F thiol probe), subsequent studies were conducted with 750 μg β-Lg/mL emulsion.

Effect of Amino Acid Residues on the Antioxidant Activity of β-Lg. Cysteine and tryptophan oxidation was observed in samples containing 750 μg β-Lg/mL emulsion over the course of the study (**Figure 4**), which is consistent with previous studies

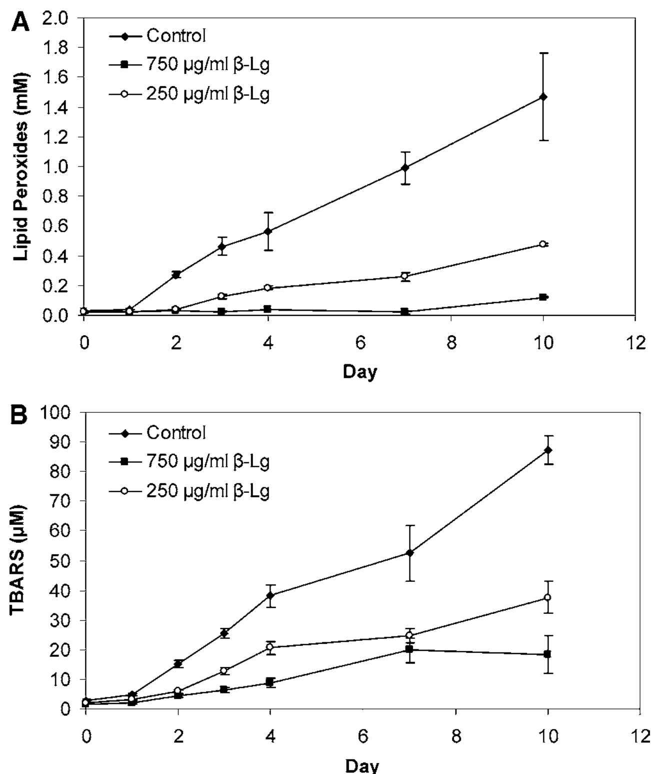

Figure 3. Effect of 250 and 750 μg/mL continuous phase β-Lg on the formation of (**A**) lipid hydroperoxides and TBARS (**B**), in washed Brij-stabilized 5% menhaden oil-in-water emulsions at pH 7.0. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

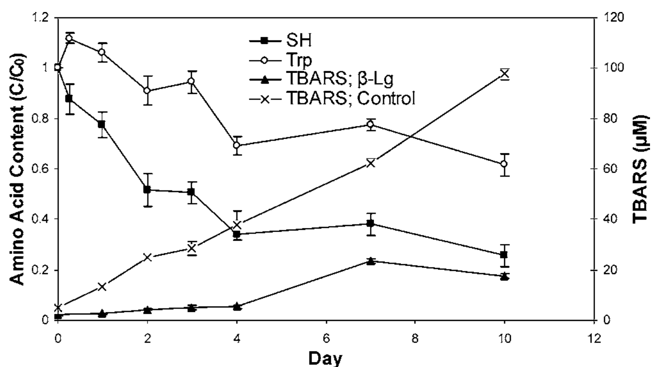

Figure 4. Decline of free sulfhydryls and tryptophan in 750 μg/mL β-Lg dispersed in the continuous phase of Brij-stabilized 5% menhaden oil-in-water emulsions at pH 7.0. Free sulfhydryl and tryptophan concentrations are expressed relative to the concentration of free sulfhydryls and tryptophan at each sampling time (C) and are compared to concentrations at day 0 (C_0). Data points represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

showing that these amino acids are free radical scavengers (*6*, *8*). It was observed that free sulfhydryl and tryptophan loss preceded the detection of lipid hydroperoxides and TBARS in the β-Lg-containing samples. A significant ($p \leq 0.05$) reduction in free sulfhydryls and tryptophan residues was observed after 1 and 4 days, respectively. After 4 days, 66 and 31% of free sulfhydryls and tryptophan residues were oxidized, respectively, and an increase in TBARS concentration was observed. After day 4, cysteine and tryptophan oxidation slowed appreciably (74 and 39% of free sulfhydryls and tryptophan residues were oxidized, respectively, at day 10), indicating that most of these amino acid residues were lost while lipid oxidation was in a

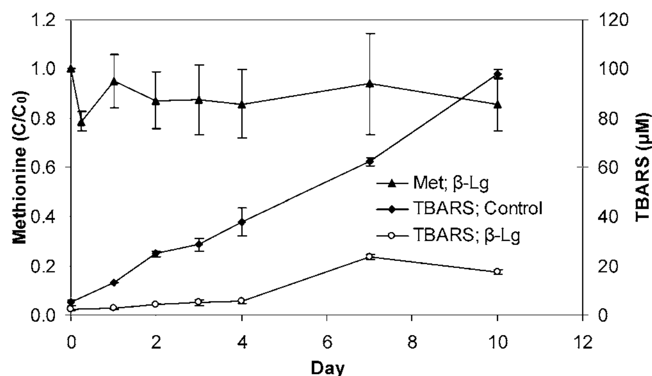

Figure 5. Decline of methionine residues in 750 μg/mL β-Lg dispersed in the continuous phase of Brij-stabilized 5% menhaden oil-in-water emulsions at pH 7.0. Methionine concentrations at each sampling time (C) are compared to concentrations at day 0 (C_0). Data points represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

lag period. The depletion of cysteine and tryptophan, which preceded the onset of lipid oxidation, suggests that these residues act as antioxidants. The fact that not all free sulfhydryls or tryptophan residues were lost is consistent with what is known about the tertiary structure of β-Lg. Cys121, the only free sulfhydryl group in β-Lg, is partially buried within the protein core (*6*), thus making it difficult for aqueous prooxidants to access this residue. Similarly, only one of β-Lg's two tryptophan residues is solvent accessible (*31*). Brownlow et al. report that the two tryptophan residues exhibit only 50% reactivity due to the observation that Trp19 is completely buried and Trp61 is surface exposed (*31*). This may explain why less than half (39%) of β-Lg's tryptophan residues were oxidized. However, it has also been observed that Trp19 does not contribute significantly to the intrinsic fluorescence of β-Lg because the guanidine group of the neighboring Arg124 residue, which lies within 3.0−4.0 Å of Trp 19, is capable of fluorescence quenching (*31*). If this were the case, then the 39% reduction in β-Lg fluorescence observed in this study is likely a consequence of Trp61 oxidation.

No significant ($p \leq 0.05$) methionine oxidation was observed in continuous phase β-Lg over the course of the study (**Figure 5**), despite its reported oxidative lability (*12*, *18*, *26*). This may suggest that β-Lg's methionine residues at pH 7 are not sufficiently surface exposed and are therefore physically unable to participate in free radical scavenging. This observation may be consistent with the reported X-ray crystal structure of β-Lg (*31*), in which it appears that all methionine residues (with the possible exception of Met7) are partially or completely buried within the protein core, and thus inaccessible to the solvent phase. In addition, it may be reasonable to assume that the concentration, location, and/or energy of the free radicals present in the emulsion were insufficient to cause extensive methionine oxidation. Levine et al. found that the surface-exposed methionine residues of glutamine synthetase were oxidized within a range of hydrogen peroxide concentrations between ∼5 and 160 mM (*18*). Hydrogen peroxide molecules can break down via homolytic cleavage (often catalyzed by transition metals) to yield extremely reactive hydroxyl radicals ($E^{\circ\prime} = 2310$ at pH 7.0); however, the reduction potential of the aliphatic alkoxyl and peroxyl radicals found in lipid systems is considerably lower ($E^{\circ\prime} = 1600$ and 1000, respectively, at pH 7.0) (*32*). It is also possible that lipid-based radicals are unable to access buried methionine residues due to steric effects since they are considerably larger than hydroxyl radicals. In addition, hydroxyl radicals, such as those used to oxidize methionine in Levine's study (*18*), are more water soluble than the radicals derived from the oxidative decomposition of lipids in the oil-in-water emulsions in this study. As a result, hydroxyl radicals are present at higher concentrations in the aqueous phase in which the protein is dispersed as compared with lipid-based radicals. These factors may help to explain why methionine oxidation was observed in a system containing a relative high concentration of hydrogen peroxide and not in the emulsion system used in this study that contained low levels of lipid hydroperoxides.

In conclusion, the presence of low concentrations of β-Lg dispersed in the continuous phase of oil-in-water emulsions was observed to inhibit lipid oxidation. Although the complete mechanism by which the antioxidant activity occurs is unclear, it appears that free radical scavenging by amino acid residues plays a crucial role. The free cysteine and tryptophan residues in β-Lg oxidize prior to emulsified menhaden oil, suggesting that they act as antioxidants. However, the methionine residues in β-Lg do not appear to participate in free radical scavenging in this system. This may be due in part to the position of the methionine residues with regard to the tertiary structure of β-Lg, the nature of the endogenous oxidant (i.e., lipid-based radicals), or the relative low oxidative stress of the system.

The application of food grade, nonsynthetic antioxidants, such as β-Lg, to oil-in-water emulsions could prove an effective and consumer friendly strategy for inhibiting lipid oxidation. Amino acid residues including cysteine, tryptophan, and methionine can be buried within the interior of proteins thus limiting their effectiveness due to their lack of accessibility to water- or lipid-soluble radicals. It is conceivable that β-Lg could behave more effectively as an antioxidant if the antioxidative amino acids in the protein's interior were exposed to the aqueous phase by denaturation or by partial hydrolysis; however, this may alter the chelation capacity of the protein, which is also important in antioxidant activity.

LITERATURE CITED

(1) Nawar, W. W. Food chemistry. In *Food Science and Technology*, 3rd ed.; Fennema, O. R., Ed.; Marcel Dekker: New York, 1996.

(2) Akoh, C. C.; Min, D. B. *Food Lipids: Chemistry, Nutrition, and Biotechnology*, 2nd revision and expanded ed.; M. Dekker: New York, 2002.

(3) Watkins, S. M.; German, J. B. Food lipids: Chemistry, nutrition, and biotechnology. In *Food Science and Technology*, 117, 2nd revision and expand ed.; Akoh, C. C., Min, D. B., Eds.; M. Dekker: New York, 2002.

(4) McClements, D. J. *Food Emulsions: Principles, Practice, and Techniques*, 2nd ed.; CRC Press: Boca Raton, 2005.

(5) McClements, D. J.; Decker, E. A. Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *J. Food Sci.* **2000**, *65* (8), 1270−1282.

(6) Tong, L. M.; Sasaki, S.; McClements, D. J.; Decker, E. A. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *J. Agric. Food Chem.* **2000**, *48* (5), 1473−1478.

(7) Hu, M.; McClements, D. J.; Decker, E. A. Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *J. Agric. Food Chem.* **2003**, *51* (5), 1435−1439.

(8) Allen, J. C.; Wrieden, W. L. Influence of milk-proteins on lipid oxidation in aqueous emulsion. 1. Casein, whey-protein and alpha-lactalbumin. *J. Dairy Res.* **1982**, *49* (2), 239−248.

(9) Viljanen, K.; Kylli, P.; Hubbermann, E. M.; Schwarz, K.; Heinonen, M. Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. *J. Agric. Food Chem.* **2005**, *53* (6), 2022−2027.

(10) Leaver, J.; Law, A. J. R.; McCrae, C. H. Chemical changes in β-lactoglobulin structure during aging of protein-stabilized emulsions. *Int. J. Food Sci. Technol.* **2000**, *34*, 503−508.
(11) Leaver, J.; Law, A. J. R.; Brechany, E. Y. Covalent modification of emulsified beta-casein resulting from lipid peroxidation. *J. Colloid Interface Sci.* **1999**, *210* (1), 207−214.
(12) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* **1997**, *10* (5), 485−494.
(13) Stadtman, E. R. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **1993**, *62*, 797−821.
(14) Sharp, J. S.; Becker, J. M.; Hettich, R. L. Analysis of protein solvent accessible surfaces by photochemical oxidation and mass spectrometry. *Anal. Biochem.* **2004**, *76* (3), 672−683.
(15) Faraji, H.; McClements, D. J.; Decker, E. A. Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *J. Agric. Food Chem.* **2004**, *52* (14), 4558−4564.
(16) Hernández-Ledesma, B.; Davalos, A.; Bartolome, B.; Amigo, L. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53* (3), 588−593.
(17) Taylor, M. J.; Richardson, T. Antioxidant activity of skim milk—Effect of heat and resultant sulfhydryl-groups. *J. Dairy Sci.* **1980**, *63* (11), 1783−1795.
(18) Levine, R. L.; Mosoni, L.; Berlett, B. S.; Stadtman, E. R. Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93* (26), 15036−15040.
(19) Brot, N.; Weissbach, H. Biochemistry and physiological role of methionine sulfoxide residues in proteins. *Arch. Biochem. Biophys.* **1983**, *223* (1), 271−281.
(20) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37* (8), 911−917.
(21) Shantha, N. C.; Decker, E. A. Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *J. AOAC Int.* **1994**, *77* (2), 421−424.
(22) McDonald, R. E.; Hultin, H. O. Some characteristics of the enzymatic lipid-peroxidation system in the microsomal fraction of flounder skeletal-muscle. *J. Food Sci.* **1987**, *52* (1), 15.
(23) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193* (1), 265−275.
(24) Hazell, L. J.; Stocker, R. Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochem. J.* **1993**, *290* (Part 1), 165−172.
(25) Carr, A. C.; Tijerina, T.; Frei, B. Vitamin C protects against and reverses specific hypochlorous acid- and chloramine-dependent modifications of low-density lipoprotein. *Biochem. J.* **2000**, *346* (Part 2), 491−499.
(26) Requena, J. R.; Groth, D.; Legname, G.; Stadtman, E. R.; Prusiner, S. B.; Levine, R. L. Copper-catalyzed oxidation of the recombinant SHa(29−231) prion protein. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98* (13), 7170−7175.
(27) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 8th ed.; Iowa State University Press: Ames, 1989; p 503.
(28) Parran, D. K.; Barker, A.; Ehrich, M. Effects of thimerosal on NGF signal transduction and cell death in neuroblastoma cells. *Toxicol. Sci.* **2005**, *86* (1), 132−140.
(29) Keowmaneechai, E.; McClements, D. J. Influence of EDTA and citrate on physicochemical properties of whey protein-stabilized oil-in-water emulsions containing $CaCl_2$. *J. Agric. Food Chem.* **2002**, *50* (24), 7145−7153.
(30) Mancuso, J. R.; McClements, D. J.; Decker, E. A. Ability of iron to promote surfactant peroxide decomposition and oxidize α-tocopherol. *J. Agric. Food Chem.* **1999**, *47* (10), 4146−4149.
(31) Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C.; Sawyer, L. Bovine beta-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure* **1997**, *5* (4), 481−495.
(32) Buettner, G. R. The pecking order of free radicals and antioxidants: Lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **1993**, *300* (2), 535−543.

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