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Antioxidant Mechanisms of Enzymatic Hydrolysates of β-Lactoglobulin in Food Lipid Dispersions

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The antioxidant activities of aqueous phase β-lactoglobulin (β-Lg) and its chymotryptic hydrolysates (CTH) were compared in this study. Proteins and peptides have been shown to inhibit lipid oxidation reactions in oil-in-water emulsions; however, a more fundamental understanding of the antioxidant activity of these compounds in dispersed food lipid systems is lacking. CTH was more effective than an equivalent concentration of β-Lg in retarding lipid oxidation reactions when dispersed in the continuous phase of Brij-stabilized oil-in-water emulsions (pH 7). Furthermore, it was observed that CTH had higher peroxyl radical scavenging and iron-binding values than β-Lg. Liquid chromatography−mass spectrometry (LC-MS) was used to measure the rate of oxidation of three oxidatively labile amino acid residues (Tyr, Met, and Phe) in certain CTH peptide fragments. Significant oxidation of specific Tyr and Met residues present in two separate 12 amino acid peptide fragments was observed in the days preceding lipid oxidation (39 and 55% of Tyr and Met were oxidized, respectively, by day 4 of the study); however, no significant oxidation of the Phe residue present in a specific 14 amino acid peptide fragment could be observed during the same time period. These data could suggest that Met and Tyr residues are capable of scavenging radical species and have the potential to improve the oxidative stability dispersed food lipids.

KEYWORDS: Lipid oxidation; β-lactoglobulin; enzymatic hydrolysates; antioxidants; mass spectrometry; food emulsions

INTRODUCTION

Fats and oils containing high concentrations of polyunsaturated fatty acids are considered to be beneficial to human health (*1*, *2*), yet their incorporation into food products is complicated by their susceptibility to oxidative deterioration (*3*, *4*). It is often necessary to employ the use of antioxidant technologies in food product formulations to inhibit lipid oxidation; however, food processors are often restricted to currently approved antioxidant additives because the cost of gaining regulatory approval for new synthetic antioxidants is prohibitive and because many additives are often not deemed to be label friendly. Therefore, the identification of effective, yet naturally derived, antioxidants could be of great interest to the food industry.

Several studies have demonstrated that oil-in-water food emulsion systems can be effective vehicles for delivering bioactive lipids (*5*−*8*) and that proteins dispersed in the continuous phase of such emulsions can inhibit deleterious oxidation reactions by scavenging free radicals and chelating prooxidative metals (*9*, *10*). It may be possible, however, to further enhance the antioxidant activity of proteins by subjecting them to hydrolysis. This can potentially increase the solvent exposure of amino acid residues that are typically buried when the protein is in its native conformation, thereby increasing their ability to scavenge free radicals and chelate prooxidant metals.

The antioxidant activity of protein hydrolysates in oxidizing lipid environments has been demonstrated in several studies. Sakanaka et al. have reported that enzymatic hydrolysates derived from common food proteins are effective antioxidants in various systems containing dispersed lipids, such as beef and tuna homogenates (*11*−*13*). The authors demonstrated that egg yolk protein hydrolysates are versatile antioxidants in a variety of in vitro assays, capable of scavenging superoxide, hydroxyl, and peroxyl radicals and inhibiting β-carotene bleaching. The same hydrolysates were able to inhibit the formation of thiobarbituric acid reactive substances (TBARS) in model food systems. Diaz and Decker also reported that caseinophosphopeptides and casein hydrolysates were effective antioxidants in both model (phosphatidylcholine liposome) and applied (cooked beef homogenate) food systems and suggested that this antioxidant activity was, in part, due to iron chelation (*14*).

Rival et al. compared the relative effectiveness of native proteins and their enzymatic hydrolysates as antioxidants in lipid

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systems in which peroxidation was initiated either by an enzyme (soybean lipoxygenase) or a nonenzymatic peroxyl radical generating system (2,2′-azobis[2-methylpropionamidine] dihydrochloride; AAPH) (*15*). The researchers demonstrated that the proteolytic digests of casein were on the order of 106−120% more effective at inhibiting AAPH-initiated lipid peroxidation than bovine serum albumin, a known free radical scavenging protein, and that the antioxidant activity of casein or β-casein was enhanced upon proteolysis. Of the digest peptides tested, those consisting of amino acid residues with hydrophobic side chains (e.g., Val and Leu) were antioxidative in oxidizing lipid environments, which may have made them preferred targets for lipophilic radical intermediates, such as peroxyl radicals derived from lipid hydroperoxides. However, subsequent studies revealed that the hydrophobicity of peptides does not fully account for their ability to inhibit lipid oxidation reactions (*15*). Thus, the authors suggest that a peptide's overall antioxidant activity is a function of primary structure (i.e., the presence of certain oxidatively active amino acids and their specific sequence in a given peptide) and/or its physicochemical properties.

Protein hydrolysates are thought to inhibit oxidation reactions by mechanisms similar to those of native protein (e.g., scavenging of oxidants and chelation of transition metals); however, little is known with regard to their activity in dispersed lipid systems. The goal of this research is to elucidate the mechanisms by which peptides inhibit oxidation reactions in foods, with particular attention given to oil-in-water emulsions. This may potentially lead to the successful development of a novel class of natural antioxidants.

MATERIALS AND METHODS

Materials. Food-grade lyophilized β-lactoglobulin was donated by Davisco Foods International, Inc. (Eden Prairie, MN). Chymotrypsin (EC 3.4.21.1; type I-S, 44 units/mg of protein) from bovine pancreas was purchased from Sigma Chemical Co. (St. Louis, MO). Unstabilized, deodorized, refined, and bleached menhaden oil (eicosapentaenoic acid, 10−17%; docosahexenoic acid, 7−12%) was obtained from Omega Protein (Houston, TX). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Fluka (St. Louis, MO). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). All other chemicals were of reagent or HPLC grade and were obtained from Sigma Chemical Co. or Fisher Scientific (Pittsburgh, PA). Distilled, deionized water was used throughout the study.

Methods. *Preparation of Hydrolysates.* Chymotryptic hydrolysates (CTH) of β-Lg (1 mg/mL) were prepared by hydrolyzing β-Lg with chymotrypsin (1:20, enzyme-to-substrate ratio, w/w) in Tris-HCl buffer (pH 8. 0) at 37 °C for 24 h (*16*). Sodium azide (0.02% w/w) was used as an antimicrobial agent. The progress of β-Lg hydrolysis was followed throughout incubation by assaying for total protein and free primary amines. Total protein was determined with the bicinchoninic acid (BCA) method using β-Lg as a standard (*17*). Free amines were determined with the TNBS method using glycine as a standard (*18*). Following incubation, hydrolysis was stopped by thermally deactivating chymotrypsin in a 95 °C water bath for 15 min, followed by cooling in a water bath at ambient temperature for 10 min. β-Lg in the absence of enzyme was used as a control and was subjected to identical incubation, thermal deactivation, and lyophilization conditions. After these treatments were completed, both β-Lg and its enzymatic hydrolysates were lyophilized. The nitrogen content of the protein and protein hydrolysates was measured by adapting the micro Dumas method in a Perkin-Elmer 240 elemental analyzer (*19*) at the University of Massachusetts Microanalysis Laboratory. All freeze-dried protein and protein hydrolysates were stored at −80 °C until use.

Peroxyl Radical Absorbance Capacity Assay. The peroxyl radical scavenging activities of β-Lg and CTH were determined using a method adapted from that of Ou et al. (*28*), as described by Diaz and Decker (*14*). A 200 mM solution of AAPH and a 50 nM solution of fluorescein in 75 mM potassium phosphate buffer at pH 7.0 were prepared for each experiment and kept on ice. For each run, fluorescein was held at 37 °C in a water bath for 15 min and then brought to a final concentration of 45 nM in a system with 0.1 mM EDTA, 20 mM AAPH, 50 μg/mL protein or chymotryptic hydrolysate, and phosphate buffer (pH 7.0). Analyses were performed on a fluorometer (excitation = 493 nm, emission = 515 nm; Hitachi F-2000, Tokyo, Japan) with the temperature maintained at 37 °C. Fluorescence was recorded every minute for 40 min, and the fluorescence relative to the initial time (F/F_0) was calculated for the fluorescence decay curve. The relative peroxyl radical absorbance capacity values of the β-Lg or CTH were calculated and compared to control samples containing either AAPH alone or fluorescein alone.

Iron Nitrilotriacetate (NTA)−Protein Binding Experiments. The ability of β-Lg and CTH to bind iron was determined using a modified method of Lin et al. (*26*). A solution of ferric iron chelated to NTA was prepared by mixing 1 volume of 0.5 M $FeCl_3$ (in 0.05 M HCl) with 2 volumes of 0.5 M NTA in water. Fe−NTA was added to 0.05 M HEPES buffer (pH 7.0) so that the final concentration of $FeCl_3$ was 1.0 mM. β-Lg or CTH (10 mL of 750 μg of sample/mL of PBS) was placed inside a dialysis bag (Spectra/Por 3 membrane, 500 molecular weight cutoff, Spectrum Laboratories Inc., Rancho Dominguez, CA) and incubated at 4 °C in 1000 mL of the Fe−NTA buffer mixture for 24 h (*26*). After dialysis, the protein concentrations inside the dialysis bags were measured using the method described by Lowry et al. (*27*).

A protein-precipitating solution was prepared with hydroxylamine hydrochloride (0.72 M), trichloroacetic acid (0.61 M), and 100 mL of 12 N HCl. To measure protein-bound iron, protein samples removed from the dialysis bags (2 mL) were mixed with 1 mL of the protein-precipitating solution and incubated at room temperature overnight. Samples were then centrifuged at 1750*g* for 10 min. The resulting supernatants (1 mL) containing iron released from the protein were mixed with 2 mL of ammonium acetate buffer (10% w/v) followed by 0.5 mL of 9.0 mM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine). Absorbance was determined at 562 nm after 1 h. The iron concentration was determined using a standard curve prepared from $FeCl_3$ (*10*).

Preparation of Emulsions. Brij 35-stabilized menhaden oil-in-water emulsions were used in all lipid oxidation studies. The emulsifier solution was prepared by dispersing 17 mM Brij 35 in phosphate-buffered saline (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 bio homogenizer (Biospec Products Inc., Bartlesville, OK) to produce a coarse 25% (w/w) oil-in-water emulsion. Emulsion droplet size was then further reduced with an ultrasonicator (Fisher Sonic Dismembrator 500, Pittsburgh, PA) at 4 °C for 3 min at 80% 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 0.60 to 0.80 μm.

Surfactant in the continuous phase of an emulsion can affect lipid oxidation reactions. Therefore, the stock emulsion (∼45 mL aliquots) was divided among three centrifuge tubes (Sorvall 75 mL, 35 × 80 mm, Asheville, NC) and centrifuged at 36000*g* at 4 °C for 60 min to cream the emulsion droplets. After separation, the continuous phase (lower layer) was removed by syringe and discarded, and an equal volume of fresh buffer was added. The emulsion was dispersed by vortexing for 2 min. The procedure of centrifugation, removal of continuous phase, and reconstitution of emulsion droplets was repeated a total of three times.

Following the final wash, the total lipid content of the emulsion was determined by modifying Bligh and Dyer's method (*20*). Lipid fractions were extracted from 300−400 mg of emulsion samples with 4 mL of methanol, 2 mL of chloroform, and 0.4 mL of water in 15 mL glass culture tubes. The samples were then stirred by vortex for 30 s and placed in an ultrasonic bath (Fisher Sonic Dismembrator 500) at 4 °C for 15 min at 40% power. Equal volumes of chloroform and water (2 mL) were then added, and the samples were stirred once more by vortex for 30 s followed by centrifugation at 3300*g* for 15 min at

ambient temperature. The upper layer (methanol/water) was removed using a Pasteur pipet, and the remaining layer was passed through a 2.5 cm thick layer of anhydrous sodium sulfate using a Whatman no. 1 filter paper and glass funnel into a preweighed glass culture tube. The solvents were then removed under nitrogen gas, and the samples were dried in an oven for 1 h at 170 °C. The sample tubes were placed in a desiccator for 30 min following drying and subsequently weighed. The total lipid content was determined by subtracting the weight of the empty glass culture tube from the weight of the container plus the isolated lipid. After determination of the fat content of the emulsion, the lipid concentration was adjusted to 5% (w/w) by dilution with β-Lg or enzymatic hydrolysates of β-Lg in fresh buffer containing 0.02% sodium azide as an antimicrobial agent. The control emulsion was diluted with buffer containing only sodium azide.

Preparation of Bulk Oils. Stock solutions of bulk menhaden oil samples were prepared by dissolving 0.01 g of β-Lg or CTH directly into 10 g of menhaden oil in glass culture tubes (Kimble Glass, 25 × 150 mm). The samples were mixed by vortex on high for 2 min, covered with Parafilm (Alcan Inc., Quebec, Canada), and then placed in a sonicating water bath for 45 min in the absence of light. The bulk oil samples were subsequently diluted with additional menhaden oil to achieve various final concentrations of β-Lg or CTH.

Lipid Oxidation Measurements. Emulsions (4 mL) and bulk oil samples (0.3 g) were placed in capped test tubes (Fisherbrand 13 × 100 mm) and allowed to oxidize in the absence of light at 20 or 37 °C. Lipid hydroperoxides were measured according to the method described by Shantha and Decker (*21*). In bulk menhaden oil containing β-Lg or CTH, the samples were diluted 20−60 times with isooctane prior to the addition of ammonium thiocyanate and ferrous sulfate depending on the extent of oxidation of the oil (*22*). In menhaden oil-in-water emulsions, the samples (0.3 mL) were mixed 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 $FeSO_4$). 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, U.K.). Hydroperoxide concentrations were determined using a standard curve prepared with cumene hydroperoxide.

The formation of secondary oxidation products was followed by measuring either TBARS or headspace aldehydes. TBARS (*23*) were determined by mixing between 0.1 and 1.0 mL (final volume adjusted to 1.0 mL with double-distilled water) of emulsion or bulk oil (diluted 20−60 times in hexadecane) with 2.0 mL of TBA reagent (15% w/v trichloroacetic 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.

The concentration of headspace aldehydes was determined by placing 1 mL of emulsion samples into 10 mL headspace vials sealed with poly(tetrafluoroethylene)/butyl rubber septa. Headspace propanal was detected using a gas chromatograph (Shimadzu 17A) equipped with a headspace sampler (Hewlett-Packard 19395A) (*24*). The headspace conditions were as follows: sample temperature, 45 °C; sample loop and transfer line temperature, 110 °C; pressurization, 10 s; venting, 10 s; injection, 1.0 min. The volatiles were separated isothermally at 70 °C on an HP methyl silicone (DB-1) fused silica capillary column (50 m, 0.31 mm i.d., 1.03 mm film thickness). The splitless injector temperature was 180 °C, and the eluted compounds were detected with a flame ionization detector at 200 °C. Concentrations were determined from peak areas using a standard curve made from authentic propanal.

Determination of Amino Acid Oxidation. Amino acid oxidation analysis was performed on continuous phase CTH free of Brij-stabilized oil droplets. To remove oil droplets, emulsions were centrifuged (36000*g* at 4 °C) for 60 min in disposable plastic Medidroppers (154 mm, 3−4 mL capacity; Fisher Scientific) placed within Sorvall centrifuge tubes containing 5 mL of water, and the collected emulsion droplets were removed and discarded by cutting the Medidropper just below the creamed lipid droplet layer. All hydrolysate solutions used for amino acid analysis were stored at −80 °C until use.

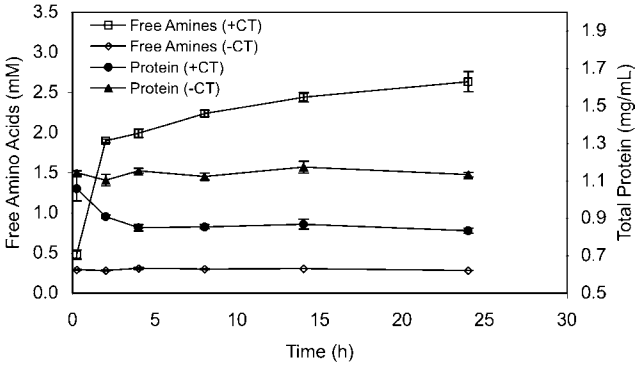

Figure 1. Increase in free amines and concomitant decrease in peptide bonds in β-Lg samples containing chymotrypsin (CT+). Control samples of β-Lg (CT−) were prepared without the enzyme. Chymotrypsin hydrolysis was carried out at 37 °C for 24 h.

The modification of specific amino acid resides in CTH was measured using mass spectrometry (MS) and liquid chromatography−mass spectrometry (LC-MS) in a manner similar to that reported previously (*25*). Aliquots were extracted and diluted with distilled, deionized water and acid (digest/water/acid = 58.5:38.5:3. 0) for analysis by direct injection electrospray ionization (ESI) MS (*25*). The chymotryptic hydrolysates were eluted with a linear gradient of acetonitrile with formic acid (0.1%) from 10% acetonitrile/water to 90% acetonitrile/water over 30 min at a flow rate of 0.5 mL/min using an HP 1100 (Agilent, Wilmington, DE) HPLC system with a C18 column (4.6 × 150 mm; Agilent) (*25*). The LC effluent was split in a 1:4 ratio with the smaller outlet being fed into the ESI source of a Bruker Esquire-LC quadrupole ion trap mass spectrometer. The needle voltage was kept at 3−4 kV, the capillary temperature was set to 250 °C, 10−60 V was applied to skimmer 1, and the capillary offset voltage was set between 20 and 60 V. Oxidized amino acid residues in peptide fragments were confirmed using MS/MS in a manner described previously (*25*).

Statistical Analysis. All experiments will be performed on triplicate samples. Statistical analysis was performed using Student's *t* test (*29*).

RESULTS AND DISCUSSION

Effect of Enzymatic Hydrolysis on Peroxyl Radical Scavenging and Chelation Capacity of β-Lg. α-Chymotrypsin hydrolyzes proteins at the carboxyl side of leucine, tyrosine, phenylalanine, tryptophan, and methionine residues (*30*). α-Chymotrypsin-catalyzed hydrolysis of β-Lg was confirmed by following the concomitant increase in free primary amines and decrease in peptide bonds as measured by the BCA method (**Figure 1**). CTH of β-Lg were found to be effective scavengers of peroxyl radicals, as determined by the oxygen radical absorbance capacity (ORAC) assay (**Figure 2**). The ability of CTH to scavenge AAPH radicals was observed to be dose dependent. After 30 min of incubation at 37 °C, fluorescein fluorescence in samples containing CTH concentrations of 2.5, 5.0, 7.5, 10, 20, and 30 μg/mL were 88, 81, 57, 32, 30, and 16% lower, respectively, compared to their initial fluorescence intensities. A control (buffer only; no added CTH) was run, and its fluorescence intensity was observed to be 3% of its initial intensity after 30 min. This compares to a control sample (buffer only; no added protein or hydrolysate) wherein fluorescein fluorescence decreased 97% after 30 min compared to its initial fluorescence value. When the peroxyl radical scavenging activities of equivalent concentrations of β-Lg or CTH (7.5 μg/mL; based on relative nitrogen content) were compared, CTH was more effective ($p \leq 0.05$) at scavenging peroxyl radicals. After 30 min of incubation, fluorescein fluorescence in samples

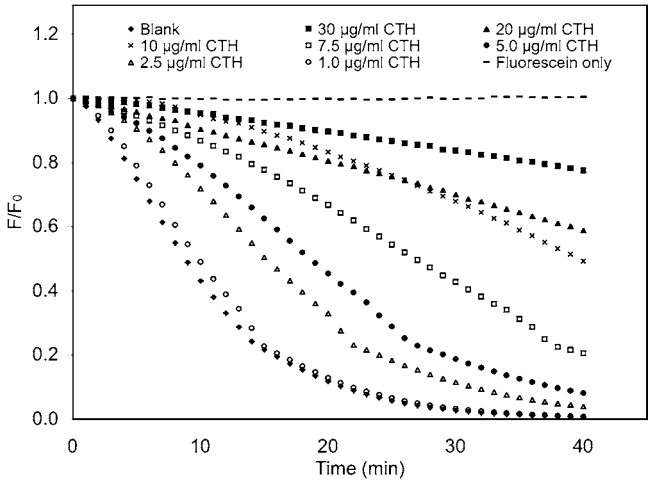

Figure 2. Changes in relative fluorescent intensity of 45 nM fluorescein (Em, λ 493 nm; Ex, λ 515 nm) in the presence of 20 mM AAPH and 0–30 μg/mL CTH (0–4.7 μg of nitrogen/mL) at 37 °C. Fluorescence values (F) are given relative to the initial time values (F_0). A control (blank) was prepared without AAPH.

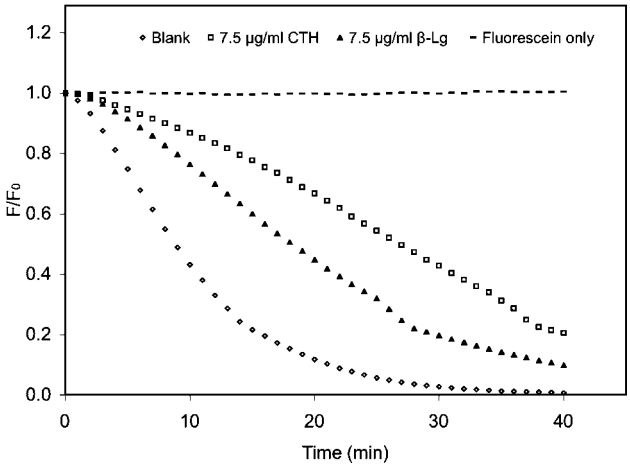

Figure 3. Effect of 7.5 μg/mL of CTH or β-Lg (1.2 μg of nitrogen/mL) on the relative fluorescent intensity of 45 nM fluorescein (Em, λ 493 nm; Ex, λ 515 nm) in the presence of 20 mM AAPH at 37 °C. Fluorescence values (F) are given relative to the initial time values (F_0). A control (blank) was prepared without AAPH.

containing CTH or β-Lg treatments was 57 and 80% lower, respectively, than the initial values (**Figure 3**).

The ability of β-Lg and CTH to bind iron was measured to assess the relative chelation capacity of each sample. Redox active transition metals such as iron are capable of promoting the decomposition of hydroperoxides and are important prooxidants in food lipids. It has been demonstrated in several studies that proteins are capable of binding aqueous transition metals in dispersed lipid systems, thus retarding lipid oxidation reactions (*10*, *14*, *31*, *32*). Protein chelators have been shown to inhibit oxidation reactions leading to rancidity by altering the physical location of prooxidant metals, forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and/or sterically hindering interactions between metals and lipid substrates such as hydroperoxides (*31*). In addition, protein chelators that do not render metals redox inactive may increase the free radical scavenging activity of proteins and peptides by localizing metals near free radical scavenging amino acids. Thus, when the metal reacts with hydroperoxides and produces free radicals, antioxidative amino acids are near the site of radical production and are more efficient antioxidants

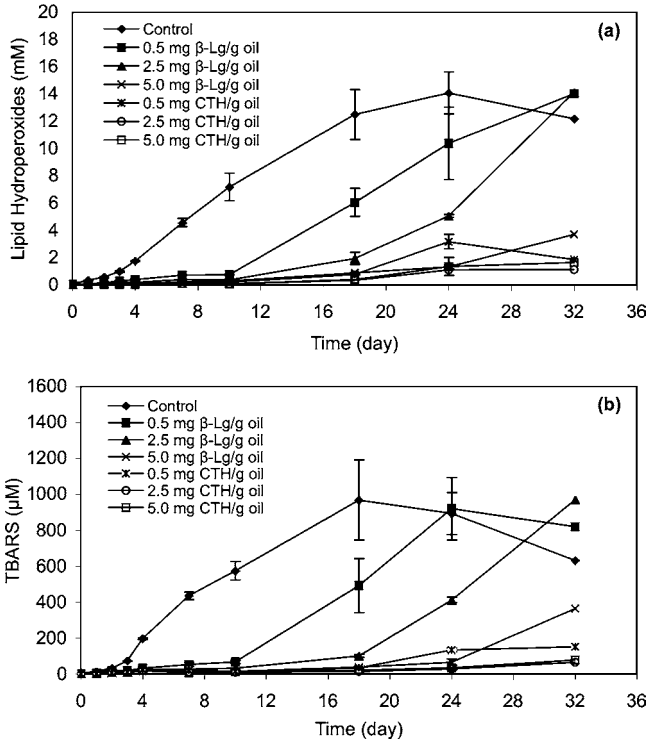

Figure 4. Influence of 0.5–5.0 mg/g of oil β-Lg or CTH (78–784 μg of nitrogen/g of oil) on the formation of (**a**) lipid hydroperoxides and (**b**) TBARS in washed Brij-stabilized 5% menhaden oil-in-water emulsions (pH 7.0) stored at 20 °C. Data points represent means ($n = 3$) ± standard deviations.

(*33*). The relative iron-binding capacities of β-Lg and its CTH were measured. Equivalent concentrations of β-Lg and CTH (7.5 μg/mL) displayed ($p \leq 0.05$) different iron chelation capacities ($p \leq 0.05$) with CTH (67.9 μmol of Fe/mg of CTH) binding approximately 3.7 times more iron than β-Lg (246 μmol of Fe/mg of β-Lg).

Antioxidant Activity of β-Lg and Its CTH in Food Lipids. CTH was observed to be a more effective antioxidant than β-Lg in an oil-in-water emulsion stored at 20 °C, which corroborates previous results that show CTH to have higher peroxyl radical scavenging activity (ORAC values) and iron chelation capacity than β-Lg. The presence of continuous phase CTH (0.5, 2.5, and 5.0 mg/g oil) significantly inhibited ($p \leq 0.05$) the formation of lipid hydroperoxides (**Figure 4a**) and TBARS (**Figure 4b**) over the course of the study. After 7 days, emulsion samples containing the lowest amount of CTH (0.5 mg of CTH/g of oil) inhibited the formation of lipid hydroperoxides and TBARS by 96 and 95%, respectively. By day 18 of the study, emulsions with 0.5 mg of CTH/g of oil were still effective in decreasing the formation of oxidation markers: lipid hydroperoxides and TBARS were inhibited by 94 and 96%, respectively. Samples containing an equivalent concentration of β-Lg (0.5 mg/g of oil) inhibited the formation of lipid hydroperoxides and TBARS by 84 and 88%, respectively, and by 52 and 49%, respectively, at day 18. The antioxidant activity of the β-Lg used in this study was slightly less than that of β-Lg used previous studies in which identical emulsion systems were used (*9*). This may be due to the fact that the β-Lg used for this study was incubated in buffer at 37 °C for 24 h and then heated at 95 °C for 15 min (the same conditions were used to prepare CTH) prior to their addition to the emulsion samples, which may have resulted in the premature oxidation of some amino acid residues (e.g., Met) or alterations in the protein's tertiary structure that negatively affected its free radical scavenging or metal chelation activity.

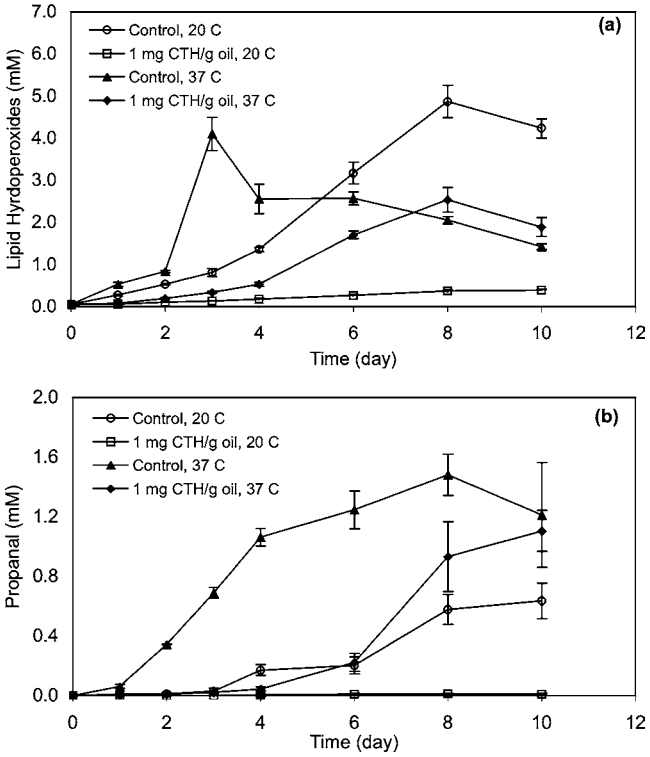

Figure 5. Effect of 1 mg/g of oil β-Lg or CTH (157 μg of nitrogen/mL) on the formation of (**a**) lipid hydroperoxides and (**b**) headspace propanal in washed Brij-stabilized 5% menhaden oil-in-water emulsions (pH 7.0) stored at either 20 or 37 °C. Data points represent means ($n = 3$) ± standard deviations.

The antioxidant activities of β-Lg and its chymotryptic hydrolysates were also evaluated in a bulk oil system. Various concentrations of β-Lg and CTH (5, 50, or 100 μg/g of oil) were dissolved directly in menhaden oil, and the capacity to inhibit lipid oxidation was measured at 37 °C. The presence of β-Lg or CTH did not significantly inhibit bulk menhaden oil oxidation compared to the control ($p \geq 0.05$) over a period of 6 days (data not shown). The reason for the ineffectiveness of β-Lg and CTH as antioxidants in a bulk oil system is unclear at this point, although it may be possible that the physicochemical properties of these compounds may not be appropriate for such a system. β-Lg is a highly water soluble protein that could partition to the aqueous interior of associated colloids, which have been proposed as potential sites of lipid oxidation in bulk oils (*22*, *34*). However, the amount of micellar water present in the bulk oil used in this study may have been so low as to be incapable of solubilizing sufficient β-Lg or CTH to inhibit oxidation reactions. Further research is required in this area to elucidate the mechanisms of protein antioxidant activity (if any) in bulk lipids.

The next objective was to monitor the oxidation of specific amino acids in CTH by mass spectrometry (MS). To optimize the detection of oxidized amino acids, oxidation conditions required sufficiently high peptide concentrations to allow for MS detection, yet enough oxidative stress to yield significant quantities of modified amino acids. **Figure 5** shows that very little lipid oxidation occurs in the presence of CTH during incubation at 20 °C, which suggests that amino acid oxidation may not be occurring. Therefore, oxidation was also performed at 37 °C in the presence of 1 mg of CTH/g of oil (**Figure 5**). In addition, headspace propanal was measured as the secondary oxidation product instead of TBARS because propanal is a more specific marker of oxidizing omega-3 fatty acids. Under these oxidation conditions, lipid hydroperoxide and headspace propanal formation remained low in emulsion samples containing 1 mg of CTH/g of oil at 20 °C throughout the course of the study (e.g., lipid hydroperoxide and headspace propanal formation were inhibited 91 and 98%, respectively, after 10 days). Samples containing 1 mg of CTH/g of oil and stored at 37 °C inhibited the formation of lipid hydroperoxides and propanal by 76 and 97% after 2 days, respectively; however, by day 10 a higher concentration of lipid hydroperoxides was detected in samples containing CTH (1.89 mM) compared to the control (1.43 mM). A high concentration of headspace propanal was also detected in CTH-containing samples (1.11 mM), albeit at a slightly lower concentration than that of the control (1.21 mM), indicating that CTH was inhibiting propanal formation by only 8.8% after 10 days of incubation. The higher concentration of lipid oxidation products in the samples stored at 37 °C indicates that oxidative stress was greater, which should result in higher concentrations of oxidatively modified amino acids.

Table 1. Amino Acid Sequence of Selected Peptide Fragments, and Their Respective *m*/*z* Ratios

oxidized residue	digest fragment	*m*/*z*	sequence
Tyr20	[Asp11–Tyr20]$^{+1}$	1180.6	DIQKVAGTW**Y**
Met24	[Ser21–Leu32]$^{+1}$	1191.6	SLA**M**AASDISLL
Phe136	[Val123–Phe136]$^{+1}$	1647.8	VRTPEVDDEALEK**F**

Oxidative Modification of Amino Acid Residues in Continuous Phase CTH As Determined by MS. The primary structures of three peptide fragments produced by chymotrypsin hydrolysis are detailed in **Table 1**. These three peptides were chosen for analysis because they each contained a single amino acid with reported oxidative susceptibility. Met24, Tyr20, and Phe136 were chosen because they were the only readily oxidized species in the digest fragments [Ser21−Leu32], [Asp11−Tyr20], and [Val123−Phe136], respectively. Other peptides with Tyr, Phe, or Met were also possibly oxidized under these conditions, but their oxidation status was difficult to confirm by MS/MS; therefore, we focused on the three peptides shown in **Table 1**. A characteristic mass increase associated with the addition of oxygen adducts ($\Delta m = +16$ for the singly charged state) can be observed when Tyr, Met, or Phe residues are oxidized (*33*); therefore, LC-MS can be used to quantify the relative abundance of specific peptide fragments of unoxidized and oxidized peptides. Oxidative modification of a given peptide fragment was determined by calculating the sum of the intensities corresponding to the oxidized species taken as a fraction of the sum of the total peptide abundance (oxidized + unoxidized species). An example of how amino acid oxidation is followed by LC-MS is given using Met24 in the peptide fragment Ser21−Leu32. **Figure 6a** depicts the selected ion chromatograms for the proteolytic fragments [Ser21−Leu32] on day 0 of the study, which contain unoxidized or oxidized Met24. A significant increase in the relative ratio of the oxidized peptide fragment ([Ser21−Leu32 + O]) to the unoxidized fragment ([Ser21−Leu32]) can be observed on day 4 (**Figure 6b**). As described under Materials and Methods, MS/MS was used to confirm that Tyr20, Met24, and Phe136 were the oxidized species in their respective peptide fragments.

The concentration of oxidized Tyr20, Met24, and Phe136 on day 0 was calculated to be 21, 7, and 12%, respectively. The fact that oxidized species could be detected at day 0 suggests that the original protein or hydrolysates were exposed to oxidative stress at some point during their preparation. Therefore, any oxidative modification occurring over days 1−8 was

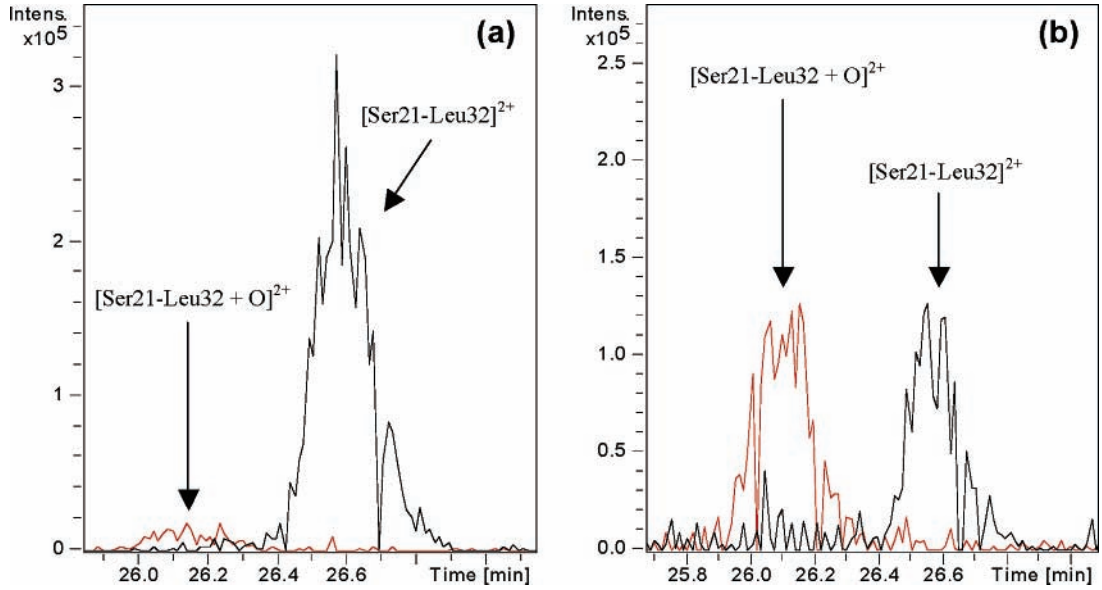

Figure 6. Selected ion chromatograms of the doubly charged digest fragments [Ser21–Leu32] (unoxidized) and [Ser21–Leu32 + O] (oxidized) on days 0 (**a**) and 4 (**b**). Chromatograms of the unoxidized and oxidized peptide fragments are overlaid.

normalized and expressed as values relative to zero time values. Lipid oxidation, as well as Tyr20 and Met24 oxidation, was observed in emulsion samples collected over the course of the study. The dispersion of CTH (1.0 mg/g of oil) within the continuous phase of emulsions inhibited the formation of lipid hydroperoxides (**Figure 7a**) and headspace propanal (**Figure 7b**) in emulsions incubated at 37 °C. By day 3 of the study, emulsions containing continuous phase CTH inhibited the formation of lipid hydroperoxides and propanal by 92 and 97%, respectively. After 4 days of incubation, the rate of lipid hydroperoxide decomposition exceeded that of formation in the control samples; however, lipid peroxidation in the emulsions containing CTH still appeared to be in lag phase at this point. An increase in the rate of formation of headspace propanal in the CTH samples was evident by day 6 of the study, whereas a significant ($p \leq 0.05$) increase in propanal in the control emulsion was observed after 1 day of storage at 37 °C. Substantial oxidative modification of Tyr20 and Met24 occurred in the days preceding the onset of lipid oxidation. After day 4, Tyr20 and Met24 were 38 and 55% oxidized, respectively, relative to their day 0 values, and 46 and 81% of these residues were oxidized by day 8. No significant oxidation of Phe136 was observed during the first 4 days of incubation, however, despite the fact that Phe, like other aromatic amino acids, is considered to be oxidatively labile. By day 8 of the study, 8% of Phe136 residues in continuous phase Val123−Phe136 peptide fragments were oxidized. This observation suggests that the Phe residue in [Val123−Phe136] is not a potentially effective antioxidant in this system because its oxidative modification could be detected only after the onset of lipid oxidation.

We reported recently that no significant Met oxidation was observed in native continuous phase β-Lg present in an identical system (i.e., Brij-stabilized 5% menhaden oil-in-water emulsion), which we attributed to a lack of solvent accessibility of the protein's Met residues (*9*). The fact that Met oxidation is now detected in continuous phase CTH could suggest that the residue is sufficiently exposed to the solvent (vs native β-Lg) to participate in oxidation reactions. The digest fragment [Ser21−Leu32] is a relatively small peptide, consisting of 12 amino acid residues, and would presumably lack the tertiary structure required for Met24 to be completely buried within a hydrophobic pocket or core. Several studies have demonstrated that Met

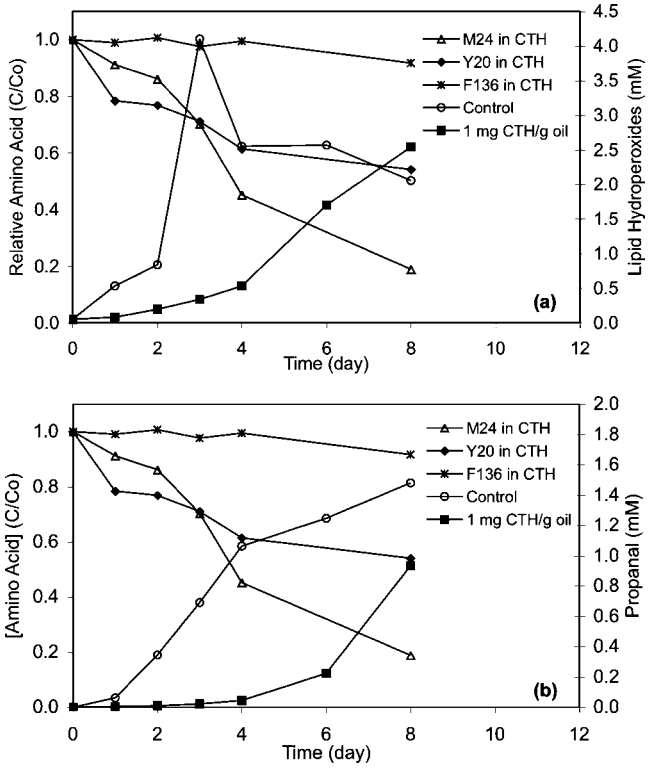

Figure 7. Formation of lipid hydroperoxides (**a**) and headspace propanal (**b**) and loss of Tyr (Y20), Met (M24), and Phe (F136) in [Asp11–Tyr20], [Ser21–Leu32], and [Val123–Phe136] peptide fragments, respectively, due to oxidative modification. Peptide fragments were dispersed in the continuous phase of washed Brij-stabilized 5% menhaden oil-in-water emulsions (pH 7.0) stored at 37 °C. Unoxidized amino acid residue concentrations (C) are expressed relative to their day 0 concentrations (C_0). Data points represent means ($n = 3$) ± standard deviations.

residues in certain proteins behave as endogenous antioxidants by quenching radical species (*35*−*37*) and reducing lipid hydroperoxides to relatively nonreactive species (e.g., lipid hydroxides) (*38*). Therefore, the fact that continuous phase CTH is a more effective antioxidant than β-Lg in the emulsion systems tested could be due, in part, to radical scavenging and/

or lipid hydroperoxide reduction by surface-exposed Met residues in CTH.

In conclusion, the results of this study confirm that the presence of continuous phase β-Lg is capable of retarding the oxidation of surfactant-stabilized oil-in-water emulsions and suggest that this protein's antioxidant activity can be enhanced with enzymatic hydrolysis. ORAC and iron-binding studies provide corroborating evidence that the chymotryptic hydrolysates of β-Lg are more effective antioxidants than native β-Lg. Furthermore, LC-MS/MS was used to assess the oxidative modification of three specific amino acid residues (Tyr20, Met24, Phe136) in CTH during the period preceding the formation of lipid oxidation products. Significant oxidation of Tyr20 in digest fragment [Asp11−Tyr20] and Met24 in [Ser21−Leu32] could be detected in the days preceding lipid oxidation (39 and 55% of Tyr20 and Met24 were oxidized by day 4, respectively, relative to their zero time values); however, no significant oxidation of Phe136 in the fragment [Val123−Phe136] was observed after 4 days. These data could suggest that Met and Tyr residues are capable of scavenging aqueous phase radical species, thereby conferring improved oxidative stability to oil-in-water food emulsions. Ultimately, a better understanding of the mechanisms governing the antioxidant activity of proteins and the enzymatic hydrolysates of proteins could result in their application as novel food antioxidants. Furthermore, specific enzymatic hydrolysates of proteins, selected on the basis of their amino acid composition (e.g., hydrolysates containing high concentrations of Met and Tyr), could be prepared for use as natural antioxidants in food lipid dispersions.

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