Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 1402–1409
Food Chemistry
www.elsevier.com/locate/foodchem

Impact of thermal processing on the antioxidant mechanisms of continuous phase β-lactoglobulin in oil-in-water emulsions

Ryan J. Elias, D. Julian McClements, Eric A. Decker *

Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, MA 01003, United States

Received 30 July 2006; received in revised form 10 November 2006; accepted 25 January 2007

Abstract

The influence of native and thermally (50–95 °C) denatured β-lactoglobulin (β-Lg) on the oxidative stability of surfactant-stabilized menhaden oil-in-water emulsions (pH 7.0) was evaluated. β-Lg (500 μg/g oil) heated at 95 °C for 30 min provided the best protection against lipid oxidation, inhibiting the formation of lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS) by 87% and 88%, respectively, following 7 days of storage. The possible mechanisms of antioxidant activity of native and heated β-Lg were evaluated by measuring peroxyl radical scavenging and iron chelating capacities of the protein treatments, as well as reactive sulfhydryl concentrations and tryptophan fluorescence (a marker of protein conformation changes). The aforementioned *in vitro* assays only partially corroborated the results from the oxidizing emulsion system since β-Lg heated at 95 °C exhibited the lowest iron chelation capacity and free sulfhydryl concentration, yet displayed the highest peroxyl radical scavenging capacity and inhibition of lipid oxidation in oil-in-water emulsions of all treatments tested. The results of this study demonstrate the feasibility of proteins as a natural class of antioxidants in food emulsions, and further elucidate the possible mechanisms by which proteins inhibit lipid oxidation.

Keywords: Lipid oxidation; Thermal denaturation; β-Lactoglobulin; Food emulsions; Antioxidants

1. Introduction

Proteins and peptides have been shown to inhibit the oxidative deterioration of lipids in a wide range of food systems including food lipid dispersions (e.g. oil-in-water emulsions) (Diaz, Dunn, McClements, & Decker, 2003; Faraji, McClements, & Decker, 2004; Hu, McClements, & Decker, 2003; Tong, Sasaki, McClements, & Decker, 2000). As a potential class of food antioxidants, proteins may represent a "natural" and low-cost alternative to synthetic food additives. The development of naturally-derived antioxidants is of great interest to food manufacturers, particularly given that consumer demand for oxidatively labile, bioactive food lipids (e.g. ω-3 fatty acids) has increased in recent years. However, before proteins can be used effectively to inhibit lipid oxidation in a wide variety of foods, a more complete understanding of the complex and multifaceted antioxidant mechanisms of these compounds is required.

Faraji and coworkers (Faraji et al., 2004) showed the importance of continuous phase (i.e. non-adsorbed) protein as antioxidants in oil-in-water emulsions, thus demonstrating that proteins can be added to the aqueous phase in order to confer oxidative stability to emulsions. Some general antioxidant mechanisms of proteins have been proposed in the literature, which may include radical scavenging by aromatic (e.g. tyrosine, tryptophan, phenylalanine) and sulfur-containing (e.g. free cysteine, methionine) amino acids, as well as chelation of endogenous transition metals (e.g. iron, copper) (Diaz & Decker, 2004; Diaz et al., 2003; Faraji et al., 2004; Levine, Berlett, Moskovitz, Mosoni, & Stadtman, 1999; Levine, Moskovitz, & Stadtman, 2000; Tong et al., 2000; Viljanen, Kivikari, & Heinonen, 2004). The preceding mechanisms are thought to be highly dependent on protein tertiary struc-

* Corresponding author. Tel.: +1 413 545 1026; fax: +1 413 545 1262.
E-mail address: edecker@foodsci.umass.edu (E.A. Decker).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.01.072

ture. For example, the ability of a protein to scavenge solvent phase radicals species is often a function of the surface exposure of its antioxidant amino acids (Levine, Mosoni, Berlett, & Stadtman, 1996). Similarly, in order for a protein to chelate aqueous metals, the amino acid residues responsible for metal binding (e.g. acidic and phosphorylated amino acids) (Baumy & Brule, 1988; Diaz & Decker, 2004) must be sufficiently solvent exposed.

Heating has been shown to increase the antioxidant activity of continuous phase skim milk in a methyl linoleate-in-water emulsion in which bovine hemoglobin was used to induce oxidation (Taylor & Richardson, 1980). Taylor and Richardson suggest that this improved activity is attributable to an increase in solvent accessibility of cysteine's sulfhydryl groups that are typically buried when the protein is in its native conformation; however, it may be possible that other oxidatively labile amino acids (e.g. methionine, tryptophan) are exposed when milk proteins are thermally modified. Evidence of the importance of free cysteine residues with regard to antioxidant activity was confirmed using the sulfhydryl blocking agent, iodoacetic acid. The authors observed that the ability of whey to inhibit lipid oxidation in emulsion was substantially reduced when its reactive sulfhydryls were blocked (Taylor & Richardson, 1980). Faraji and coworkers provided corroborating evidence that whey's sulfhydryl groups contribute to the protein's overall antioxidant activity (Faraji et al., 2004). However, the increase in reactive sulfhydryls that occurred concomitantly with protein denaturation could not fully account for the increased antioxidant activity of heated skim milk. Taylor and Richardson observed that skim milk supplemented with free cysteine did not significantly increase its antioxidant activity, despite the fact that the effective concentration of free sulfhydryls was increased. Furthermore, when skim milk was treated with sodium borohydride (a reducing agent), a 5× increase in sulfhydryl concentration was observed while the protective index of the protein decreased.

The goal of this research was to assess the ability of continuous phase proteins, at various stages of denaturation, to influence the oxidative stability of food oil-in-water emulsions containing ω-3 fatty acids. In order to effectively utilize proteins as food antioxidants in a variety of complex food matrices, a more fundamental understanding of the factors involved in protein antioxidant activity is necessary. Ultimately, this research may lead to the identification or design of proteins for use as naturally-derived food additives in emulsions containing oils susceptible to oxidation.

2. Materials and methods

2.1. Materials

Food-grade lyophilized β-lactoglobulin 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 (ADB-F) was obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of reagent or HPLC grade, and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. Methods

2.2.1. Preparation of protein solutions

Thermally modified β-Lg was prepared by heating the protein (0.5 mg protein/ml) in phosphate-buffered saline (PBS; 10 mM phosphate/0.15 M NaCl, pH 7.0) in a water bath at 50, 70, or 95 °C for 15 min, followed by cooling in a water bath at 20 °C for 30 min. An untreated protein control was prepared at the same time, and was allowed to stand in a water bath at 20 °C for 30 min. The heated protein solutions were held at 4 °C for 1 h prior to their incorporation within the emulsions, or use in either fluorescence or metal chelation assays. No insoluble protein aggregates were observed in any of the heated samples.

2.2.2. 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 17 mM Brij 35 in PBS 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 homogenizer (Biospec Products Inc., Bartlesville, OK) to produce a 25% (w/w) oil-in-water coarse emulsion. Emulsion droplet size was then further reduced with an ultra-sonicator (Fisher Sonic Dismembrator 500; Pittsburgh, PA) at 4 °C for 3 min at 80% power and 0.5 duty cycle.

2.2.3. Removal of continuous phase surfactant

Excess Brij 35 in the continuous phase of the oil-in-water emulsion was removed because surfactants have been shown to affect protein conformation and interfere with the fluorescence assays (De, Girigoswami, & Das, 2005; Elias, McClements, & Decker, 2005). To remove excess Brij, the stock emulsion (~45 ml aliquots) was divided among three centrifuge tubes (Sorvall 75 ml, 35 × 80 mm; Asheville, NC) and centrifuged at 36,000×*g* at 4 °C for 60 min. After separation, the continuous phase (lower layer) was removed with a syringe and discarded, and an equal volume of fresh PBS was added. The emulsion was redispersed by vortexing for 2 min. This 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 using a modification of Bligh and Dyer's method (Bligh & Dyer, 1959). The emulsion was then adjusted to a lipid concentration of 5% (w/w) by diluting with native or heated (50, 70, or 95 °C treated) β-Lg in fresh buffer (final protein concentration of 50, 250, or 500 μg protein/g of oil) containing sodium azide (final concentration of 0.02% w/w) as an antimicrobial agent. The

control emulsion was diluted with buffer containing only sodium azide (0.02% w/w). A laser light scattering instrument (Coulter LS-230; Miami, FL) was used to measure the particle size distribution. The mean particle size of the emulsion ranged from 0.55 to 0.70 μm and did not change during the storage studies.

2.2.4. Lipid oxidation measurements

Emulsions (1.5 ml) were placed in capped test tubes (Fisherbrand 13 × 100 mm) and allowed to oxidize in the absence of light at 20 °C for up to 11 days. Lipid hydroperoxides were measured according to Shantha and Decker (Shantha & Decker, 1994) 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 $FeSO_4$). After 20 min, the absorbance of the solutions was measured at 510 nm using an Amersham Pharmacia Biotech Ultrospec 3000 Pro scanning spectrophotometer (Cambridge, England). Hydroperoxide concentrations were determined using a standard curve prepared with cumene hydroperoxide.

Thiobarbituric acid reactive substances (McDonald & Hultin, 1987) 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 tricholoracetic 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.

2.2.5. Determination of the effect of heat on cysteine and tryptophan exposure in β-Lg

Tryptophan residues in native and heated β-Lg were measured directly by fluorescence (excitation = 280 nm, emission = 331 nm) on a Hitachi F-2000 fluorometer (Tokyo, Japan) with the cell temperature maintained at 37 °C following dilution in PBS (final concentration 100 μg protein/ml) (Hazell & Stocker, 1993). Free cysteine residues in the native and heated protein samples were derivatized with ABD-F and measured directly by fluorescence (excitation = 365 nm, emission = 492 nm) following dilution with phosphate buffer (100 mM phosphate buffer, pH 8.0, and 1 mM diethylenetriaminepentaacetic acid) to achieve a final concentration of 100 μg protein/ml (Carr, Tijerina, & Frei, 2000). The fluorometer's sample cell was maintained at 37 °C. The ABD-F thiol probe was added (10 μl of 10 mM ABD-F in PBS) to 1.0 ml of the diluted β-Lg solutions, mixed by vortex, incubated in a 60 °C water bath for 20 min prior to measurement.

2.2.6. Peroxyl radical absorbance capacity assay

The peroxyl radical scavenging activity (Ou, Hampsch-Woodill, & Prior, 2001) of native and heated β-Lg were determined using a method adapted from Ou and coworkers, as described by previously (Diaz & Decker, 2004). A 200 mM solution of 2,2′-azo-bis(2-methylpropionamidine)dihydrochloride (AAPH) and a 50 nM solution of fluorescein in 75 mM potassium phosphate buffer at pH 7.0 was 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, 30 μg protein/ml, and phosphate buffer (pH 7.0). Analysis was performed on a Hitachi F-2000 (Tokyo, Japan) fluorometer (excitation = 493 nm, emission = 515 nm) with the temperature maintained at 37 °C. Fluorescence was recorded every minute for 40 min, and the fluorescence relative to the initial time was calculated for the fluorescence decay curve. The relative peroxyl radical absorbance capacity values of the protein samples was calculated and compared to control samples containing AAPH alone.

2.2.7. Iron nitrilotriacetate–protein binding experiments

The ability of native and heated β-Lg to bind iron was determined using a modified method as described previously (Lin, Mason, Woodworth, & Brandts, 1991). To maintain iron solubility, a solution of ferric iron chelated to nitrilotriacetate (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. Solutions of native or heated β-Lg (10 ml of 500 μg protein/ml PBS) were placed inside a dialysis bag (Spectra/Por 3 Membrane, 3500 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 (Lin et al., 1991). After dialysis, the protein concentrations inside the dialysis bags were measured using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

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 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 $FeCl_3$ (Faraji et al., 2004).

2.2.8. Statistical analysis

All experiments were performed on triplicate samples. Statistical analysis was performed using Student's *t* test (Snedecor & Cochran, 1989).

3. Results and discussion

3.1. Antioxidant activity of heated β-Lg in oil-in-water emulsions

The ability of 50 and 500 μg protein/g oil β-Lg (native and heated treatments) to inhibit lipid oxidation upon addition to the continuous phase of a Brij 35-stabilized menhaden oil-in-water emulsion is shown in Figs. 1 and 2, respectively. At 50 μg protein/g oil, all protein treatments inhibited both lipid hydroperoxide and TBARS formation after 2 days of storage ($P \leqslant 0.05$). Thermal denaturation of β-Lg (50–95 °C) was not observed to increase the inhibition of either lipid hydroperoxide or TBARS formation compared to undenatured, native β-Lg when the protein was added at 50 μg protein/g oil (Fig. 1). After 7 days of storage, inhibition of lipid hydroperoxides and TBARS by heated and unheated β-Lg (50 μg protein/g oil) ranged from 6.7% to 32% and 20% to 33%, respectively, compared to the control (no added protein).

Increasing continuous phase β-Lg concentrations to 500 μg protein/g oil increased inhibition of both lipid hydroperoxide and TBARS compared to β-Lg at 50 μg protein/g oil (Fig. 2). After 7 days of storage, emulsions containing 500 μg of native, unheated β-Lg/g oil inhibited lipid hydroperoxide and TBARS formation by 59% and 49%, respectively, compared to the control (no added protein). At the concentration of 500 μg protein/g oil, heat treatment at 50 or 70 °C did not increase the ability of the continuous phase β-Lg to inhibit either lipid hydroperoxide or TBARS formation. However, thermal treatment at 95 °C did increase the ability of β-Lg to inhibit lipid oxidation: lipid hydroperoxide and TBARS concentrations were 87% and 88% lower than the control, respectively, after 7 days of storage.

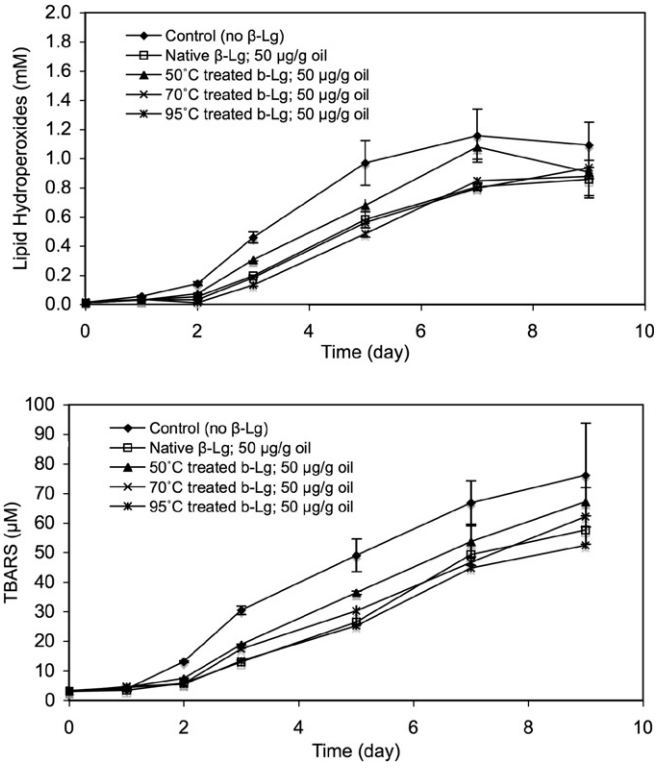

Fig. 1. Influence of 50 μg protein/g oil of native, 50, 70, or 95 °C heated β-Lg on the formation of (a) lipid hydroperoxides and (b) TBARS in washed Brij-stabilized 5% menhaden oil-in-water emulsions (pH 7.0) stored at 20 °C. Data points represent means ($n = 3$) ± standard deviations.

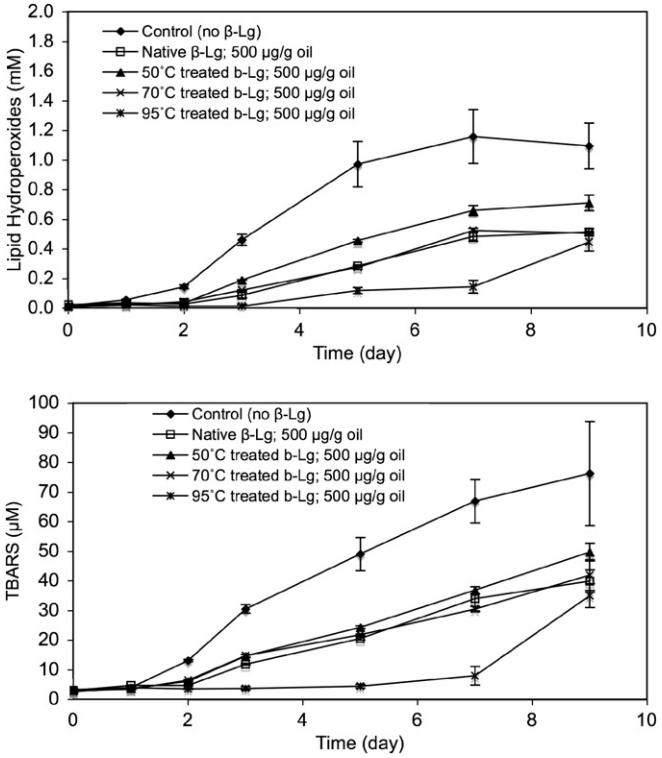

Fig. 2. Influence of 50 μg protein/g oil of native, 50, 70, and 95 °C heated β-Lg on the formation of (a) lipid hydroperoxides and (b) TBARS in washed Brij-stabilized 5% menhaden oil-in-water emulsions (pH 7.0) stored at 20 °C. Data points represent means ($n = 3$) ± standard deviations.

3.2. Effect of heat treatment on the antioxidant mechanisms of β-Lg

Trace levels of iron are common in food products (McClements & Decker, 2000), and are capable of promoting lipid oxidation through the reduction of hydroperoxides into free radical species. Therefore, additives that are capable of interfering with the prooxidant activity of iron (e.g. metal chelators) are frequently used to inhibit oxidation reactions in food lipids. It has been demonstrated in several studies that proteins are capable of binding aqueous transition metals in dispersed lipids, thereby retarding lipid oxidation reactions (Diaz & Decker, 2004; Diaz et al., 2003; Faraji et al., 2004; Tong et al., 2000). Protein chelators have been shown to inhibit reactions that lead to oxidative rancidity by reducing the chemical reactivity of transition metals, forming insoluble metal complexes, altering the physical location of prooxidant metals (e.g. increas-

ing partitioning of iron away from the lipid), and/or reducing interactions between metals and lipid substrates (e.g. hydroperoxides) through steric hindrance (Diaz et al., 2003). It has also been reported that metal-binding proteins may serve to retard oxidation reactions by binding prooxidant metals within close proximity of radical scavenging amino acids. In such a case, the antioxidant activity of the proteins is enhanced because the formation of radical species occurs within close proximately to high concentrations of radical scavenging amino acids, thus increasing radical scavenging efficiency and reducing the propensity of the free radical to migrate to, and interact with, emulsified lipids.

An iron nitriloacetate–protein-binding assay (Lin et al., 1991) was used to measure the ability of β-Lg (native and all heat treatments) to bind iron. It was observed that when β-Lg was heated at 50 °C, a slight, albeit significant ($P \leqslant 0.05$), increase in iron binding capacity occurred versus the native protein (Fig. 3). No significant change ($P \leqslant 0.05$) in chelation capacity was observed for the 70 °C heated β-Lg treatment compared to native β-Lg. However, heating β-Lg at 95 °C did significantly ($P \leqslant 0.05$) affect chelation capacity reducing iron binding 39% compared to the native protein.

The antioxidant activity of proteins is also related to the surface exposure of its radical scavenging amino acid residues (Levine et al., 1996). In the case of native β-Lg, the majority of its oxidatively labile amino acids are buried and thus inaccessible to aqueous phase radical species. Table 1 details the solvent accessibility of the major oxidatively labile amino acids in native β-Lg calculated using an algorithm for determining solvent accessible surface area, as described previously (Fraczkiewicz & Braun, 1998). Residues are considered to be solvent exposed if the ratio of their side-chain surface area to random coil value exceeds 50%, and to be buried if the ratio is less than 20%. The random coil value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations (Fraczkiewicz & Braun, 1998). As shown in Table 1, 17 of the 20 oxidatively labile amino acids in native β-Lg are solvent inaccessible while tyrosine 20, tryptophan 61 and histidine 146 are considered partially exposed. Therefore, thermal denaturation of β-Lg may expose oxidatively labile amino acids to the solvent where they can interact with free radical species.

Table 1
The solvent accessible surface area (SASA) of β-Lg's oxidatively labile amino acid residues calculated using the program GETAREA (Fraczkiewicz et al., 1998)

Residue	Sidechain	Random coil	Ratio (%)	In/out
Met 7	25.58	158.3	16.2	i
Trp 19	0.00	224.6	0.0	i
Tyr 20	53.55	193.1	27.7	
Met 24	0.00	158.3	0.0	i
Tyr 42	16.58	193.1	8.6	i
Trp 61	93.04	224.6	41.4	
Cys 66	**10.51**	**102.3**	**10.3**	**i**
Phe 82	1.58	180.1	0.9	i
Tyr 99	35.28	193.1	18.3	i
Tyr 102	13.81	193.1	7.2	i
Phe 105	16.40	180.1	9.1	i
Cys 106	**2.45**	**102.3**	**2.4**	**i**
Met 107	7.34	158.3	4.6	i
Cys 119	**0.15**	**102.3**	**0.1**	**i**
Cys 121	0.00	102.3	0.0	i
Phe 136	0.10	180.1	0.1	i
Met 145	15.17	158.3	9.6	i
His 146	43.60	154.6	28.2	
Phe 151	12.67	180.1	7.0	i
Cys 160	**12.38**	**102.3**	**12.1**	**i**

The ratio of side-chain surface area to random coil value of each amino acid residue is listed in the 4th column. The "random coil" value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations. Residues are considered to be solvent exposed if the side-chain surface area to random coil value ratio value exceeds 50% and to be buried if the ratio is less than 20% (Fraczkiewicz & Braun, 1998). Buried residues are denoted with an "i". Cys residues in bold typeface represent residues involved in disulfide bonds (i.e. cystine). A probe radius of 1.4 Å was used.

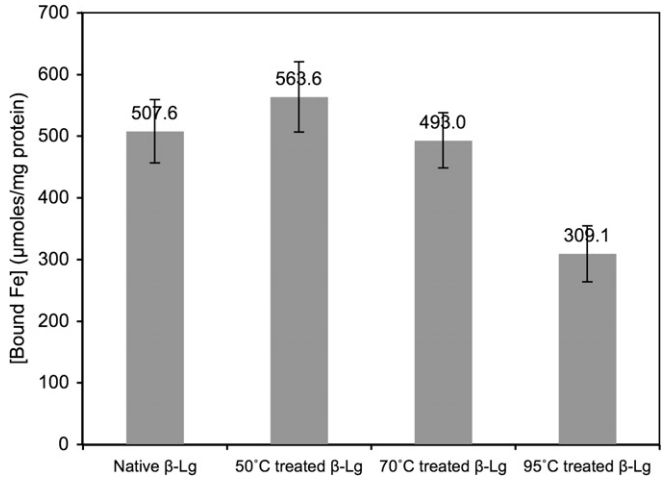

Fig. 3. Ability of native and heated β-Lg (50, 70, and 95 °C) to bind iron following incubation for 24 h at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

The thermal denaturation of β-Lg has been reported to occur in multiple stages, with an unfolding step (70–75 °C) preceding an aggregation step (78–82.5 °C) (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). In order to track temperature induced conformational change, changes in the fluorescence of tryptophan and the ability of the fluorescent probe ABD-F to react with cysteine were monitored. Tryptophan fluorescence is affected by protein tertiary structure, and therefore can be used as an intrinsic probe for detecting conformational change (Suzuki & Kanazawa, 1995). Furthermore, an increase in fluorescence could mean that a greater concentration of tryptophan residues are more surface exposed. An increase in β-Lg–ABD-F probe reactivity indicates that more sulfhydryl groups are accessible to the probe during the assay, and by extension could be more accessible to aqueous phase prooxidants in an oxidizing oil-in-water emulsion system.

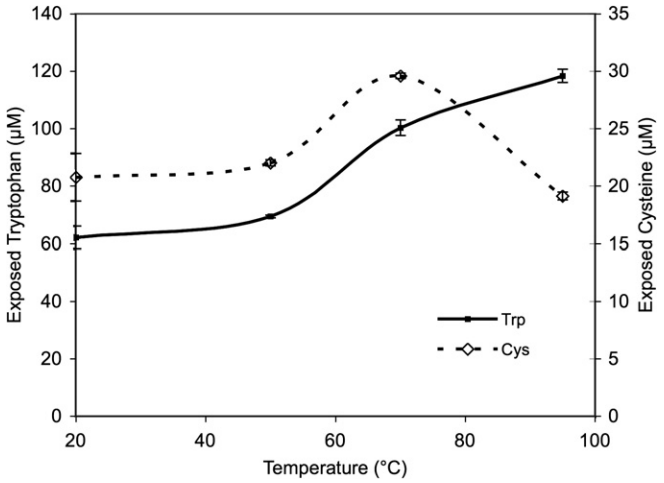

Fig. 4. Change in reactive sulfhydryl groups of endogenous cysteine residues, as well as change in tryptophan fluorescence, in β-Lg following various heat treatments (50, 70, and 95 °C). Data points represent means ($n = 3$) ± standard deviations.

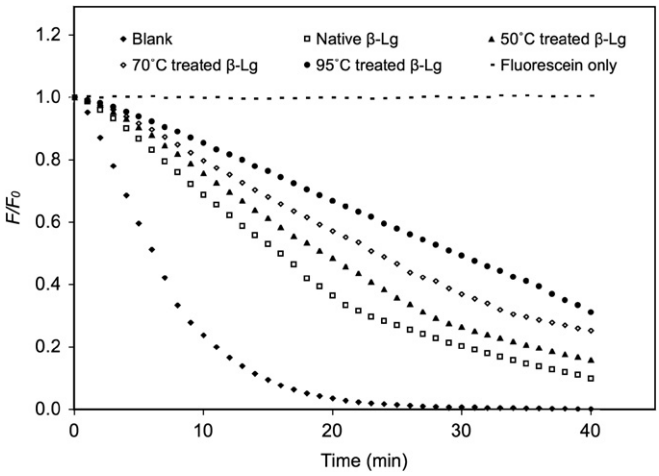

Fig. 5. Effect of 30 μg protein/ml of native, 50, 70, or 95 °C heated β-Lg on the relative fluorescent intensity of 45 nM fluorescein (Em. λ 493 nm, Ex. λ 515 nm) in the presence of 20 mM AAPH during incubation at 37 °C. Fluorescence values (F) are given relative to the initial time values (F_0). A control (blank) was prepared with AAPH and no protein.

A significant increase ($P \leqslant 0.05$) in both reactive sulfhydryls and tryptophan fluorescence was observed as β-Lg was heated from 20 to 70 °C (Fig. 4). Heating β-Lg at 50 °C resulted in a 6% and 12% increase in reactive cysteine and tryptophan fluorescence, respectively, indicating that the protein's tertiary structure has been disrupted to some degree. β-Lg heated at 70 °C resulted in a substantial increase of surface exposed cysteine and tryptophan fluorescence, which were increased by 43% and 62%, respectively compared to native (20 °C) protein. For 95 °C treated β-Lg, the fluorescence of tryptophan was 91% higher than that of native β-Lg. However, reactive sulfhydryls were observed to decrease by 8% (compared to the unheated protein) when the temperature was increased from 70 to 90 °C. This decrease suggests that excessive heat may have resulted in the formation of intermolecular disulfide bonds between free cysteine residues making them unreactive to the fluorescent probe, or that the protein had entered a second stage of protein conformational change where β-Lg's free sulfhydryl became inaccessible to the probe. Taylor and Richardson observed a similar trend in heated milk proteins, in which the concentration of reactive sulfhydryls increased when raw skim milk was heated at 90 and 110 °C for 1 min, but decreased considerably when heated at 110 °C for 30 min (Taylor & Richardson, 1980).

While fluorescence can be used to monitor the changes in the physical environment of cysteine and tryptophan upon changes in protein conformation, it does not give a direct indication that the change in protein structure increases the ability of amino acids to scavenge free radicals. Therefore, the ability of native and heated β-Lg to interact with free radicals was determined using the ORAC assay which determined the ability of compounds to scavenge peroxyl radicals generated from AAPH, and thus inhibit the fluorescence decay of fluorescein (Ou et al., 2001). It was observed that the ability of β-Lg to scavenge peroxyl radicals increased as a function of heat treatment (e.g. 95 °C treated β-Lg > 70 °C treated β-Lg > 50 °C treated β-Lg > native β-Lg) (Fig. 5). After 30 min of incubation at 37 °C, fluorescein fluorescence in samples containing equivalent concentrations (30 μg protein/ml assay) of native, 50, 70, and 95 °C treated β-Lg were 80%, 74%, 63%, and 51% lower, respectively, compared to their initial fluorescence values. This compares to a control sample (buffer only; no added protein) wherein fluorescein fluorescence decreased 99.3% after 30 min compared to its initial fluorescence values. These data indicate that the 95 °C treated β-Lg was the most effective at inhibiting the ability of peroxyl radical to decompose fluorescein.

The observed iron binding capacities, tryptophan and cysteine exposure, and peroxyl radical scavenging activity of thermally processed β-Lg treatments were only partially predictive of its antioxidant behavior in the continuous phase of oil-in-water emulsions. β-Lg (500 μg protein/g oil) heated at 95 °C exhibited the highest peroxyl radical scavenging activity, tryptophan exposure and was the most effective of all protein treatments at inhibiting the formation of lipid hydroperoxides and TBARS when added to the continuous phase of a menhaden oil-in-water emulsion. However, the iron binding capacity and concentration of reactive sulfhydryl groups were lowest for β-Lg heated at 95 °C (versus all other treatments, including the native protein). These results could be due to the fact that a greater amount of the 95 °C treated β-Lg's free radical scavenging amino acids were solvent accessible (as evidenced by increased tryptophan exposure), thus allowing the heated protein to more effectively inactivate free radicals (e.g. higher peroxyl radical scavenging activity) and inhibit lipid oxidation. Since the fluorescence of tryptophan, an oxidatively labile amino acid (Decker, Ivanov, Zhu, & Frei, 2001; Simat & Steinhart, 1998; Viljanen et al., 2004),

increased upon heating at 95 °C it is possible that other, previously buried residues (e.g. methionine, tyrosine, phenylalanine) that are potential radical scavengers also became more solvent accessible in the heated protein. In addition, it is also possible that heat induced denaturation, which has been observed to increase the overall hydrophobicity of β-Lg (Kim, Cornec, & Narsimhan, 2005; Sava et al., 2005), allows more of the protein to concentrate at the surface of the emulsion droplets where it could more effectively scavenge free radicals produced upon the decomposition of fatty acid hydroperoxides (McClements & Decker, 2000). Absorption of denatured β-Lg to the emulsion droplet would be dependent on its surface activity in relation to Brij 35 since both surface active compounds would be competing for absorption to the lipid surface.

Increased free radical scavenging activity could alone explain why the heated protein was more active, however it is also possible that due to the low concentrations of endogenous, trace iron present in the emulsion, the 95 °C heated β-Lg could still bind sufficient iron (despite its impaired chelation capacity) to retard metal-catalyzed lipid oxidation, meaning that it could be acting as both a chelator and free radical scavenger. β-Lg heated at 70 °C had increased peroxyl radical scavenging activity and solvent exposed sulfhydryl and tryptophan concentrations compared to native protein, yet it was not more effective at inhibiting lipid oxidation in the menhaden oil-in-water emulsion. This suggests that dramatic changes in protein conformation are needed to expose the amino acids that can effectively scavenge free radicals and increase antioxidant activity.

4. Conclusions

The results of this study suggest that the overall antioxidant activity of continuous phase proteins derive from a number of either competing or synergistic factors (e.g. scavenging of peroxyl radicals and chelation of transition metals). Continuous phase β-Lg heated at 95 °C (500 μg/g oil) provided the best protection against lipid oxidation in an oxidizing oil-in-water emulsion systems (pH 7.0), despite having lower iron binding values and concentrations of reactive sulfhydryls. We hypothesize that the observed enhancement in antioxidant activity of thermally denatured β-Lg (95 °C heat, 15 min) results from improved solvent accessibility of radical scavenging amino acids, and/or changes in β-Lg's surface activity, leading to improved access of the protein to free radicals. Overall, food proteins may represent a novel class of natural antioxidants that will enable food manufacturers to produce oxidatively stable, label-friendly emulsions.

Acknowledgement

This research was partially funded by the United States Department of Agriculture (NRI CSREES Grant 2004-02422).

References

Baumy, J. J., & Brule, G. (1988). Effect of pH and ionic-strength of the binding of bivalent-cations to b-casein. *Lait, 68*, 409–417.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911–917.

Carr, A. C., Tijerina, T., & Frei, B. (2000). Vitamin C protects against and reverses specific hypochlorous acid- and chloramine-dependent modifications of low-density lipoprotein. *Biochemical Journal, 346*(Pt 2), 491–499.

De, S., Girigoswami, A., & Das, S. (2005). Fluorescence probing of albumin-surfactant interaction. *Journal of Colloid and Interface Science, 285*(2), 562–573.

Decker, E. A., Ivanov, V., Zhu, B. Z., & Frei, B. (2001). Inhibition of low-density lipoprotein oxidation by carnosine histidine. *Journal of Agricultural and Food Chemistry, 49*(1), 511–516.

Diaz, M., & Decker, E. A. (2004). Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. *Journal of Agricultural and Food Chemistry, 52*(26), 8208–8213.

Diaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 51*(8), 2365–2370.

Elias, R. J., McClements, D. J., & Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase b-lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 53*(26), 10248–10253.

Faraji, H., McClements, D. J., & Decker, E. A. (2004). Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 52*(14), 4558–4564.

Fraczkiewicz, R., & Braun, W. (1998). Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *Journal of Computational Chemistry, 19*(3), 319–333.

Hazell, L. J., & Stocker, R. (1993). Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochemical Journal, 290*(Pt 1), 165–172.

Hu, M., McClements, D. J., & Decker, E. A. (2003). Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 51*(5), 1435–1439.

Kim, D. A., Cornec, M., & Narsimhan, G. (2005). Effect of thermal treatment on interfacial properties of b-lactoglobulin. *Journal of Colloid and Interface Science, 285*(1), 100–109.

Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L., & Stadtman, E. R. (1999). Methionine residues may protect proteins from critical oxidative damage. *Mechanisms of Ageing and Development, 107*(3), 323–332.

Levine, R. L., Moskovitz, J., & Stadtman, E. R. (2000). Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. *International Union of Biochemistry and Molecular Biology Life, 50*(4–5), 301–307.

Levine, R. L., Mosoni, L., Berlett, B. S., & Stadtman, E. R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proceedings of the National Academy of Science US, 93*(26), 15036–15040.

Lin, L. N., Mason, A. B., Woodworth, R. C., & Brandts, J. F. (1991). Calorimetric studies of the binding of ferric ions to ovotransferrin and interactions between binding sites. *Biochemistry, 30*(50), 11660–11669.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

McClements, D. J., & Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science, 65*(8), 1270–1282.

McDonald, R. E., & Hultin, H. O. (1987). Some characteristics of the enzymatic lipid-peroxidation system in the microsomal fraction of flounder skeletal-muscle. *Journal of Food Science, 52*(1), 15.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626.

Sava, N., Van der Plancken, I., Claeys, W., & Hendrickx, M. (2005). The kinetics of heat-induced structural changes of b-lactoglobulin. *Journal of Dairy Science, 88*(5), 1646–1653.

Shantha, N. C., & Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International, 77*(2), 421–424.

Simat, T. J., & Steinhart, H. (1998). Oxidation of free tryptophan and tryptophan residues in peptides and proteins. *Journal of Agricultural and Food Chemistry, 46*(2), 490–498.

Snedecor, G. W., & Cochran, W. G. (1989). *Statistical methods*. Ames: Iowa State University Press.

Suzuki, H., & Kanazawa, T. (1995). The tryptophan fluorescence change upon conformational transition of the phosphoenzyme intermediate in sarcoplasmic-reticulum Ca^{2+}-atpase is revealed in the absence of K^{+} and the presence of lasalocid. *Journal of Biological Chemistry, 270*(7), 3089–3093.

Taylor, M. J., & Richardson, T. (1980). Antioxidant activity of skim milk – effect of heat and resultant sulfhydryl-groups. *Journal of Dairy Science, 63*(11), 1783–1795.

Tong, L. M., Sasaki, S., McClements, D. J., & Decker, E. A. (2000). Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry, 48*(5), 1473–1478.

Viljanen, K., Kivikari, R., & Heinonen, M. (2004). Protein–lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *Journal of Agricultural and Food Chemistry, 52*(5), 1104–1111.