Mechanisms of the Antioxidant Activity of a High Molecular Weight Fraction of Whey

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The antioxidant mechanisms of whey proteins in a Tween 20-stabilized salmon oil-in-water emulsion were investigated. The antioxidant activity of the high molecular weight (HMW) fraction of whey from pasteurized milk was found to increase with concentration, as determined by its ability to inhibit TBARS and lipid peroxide formation. The ability of sulfhydryl-blocked whey to inhibit TBARS formation was reduced 60% compared to the HMW fraction alone at 7 days of storage. HMW fraction was able to scavenge peroxyl radicals, with scavenging decreasing approximately 20% when sulfhydryls were blocked. HMW fraction was able to chelate iron away from the surface of negatively charged BSA-stabilized emulsion droplets, indicating that the whey proteins were able to chelate iron. A better understanding of the mechanisms by which whey proteins inhibit lipid oxidation could increase the use of whey proteins as food antioxidants.

Keywords: *Lipid oxidation; fish oil; whey protein; antioxidants; iron; chelation*

INTRODUCTION

Whey proteins are common emulsifying, gelling, and bulking ingredients in foods and have previously been shown to have antioxidant activity (Allen and Wrieden, 1982a,b; Colbert and Decker, 1991; Donnelly et al., 1998; Ostdal et al., 1996; Taylor and Richardson, 1980). Possible antioxidant mechanisms of whey include chelation of transition metals by lactoferrin (Gutteridge et al., 1981) and serum albumin (Meucci et al., 1991) and free radical scavenging by amino acids such as tyrosine and cysteine (Wayner et al., 1987; Ostdal et al., 1996; Taylor and Richardson, 1980).

Taylor and Richardson (1980) were the first to explore the antioxidant activity of heated skim milk and milk fractions for their antioxidant activity in a methyl linoleate emulsion where oxidation was promoted by hemoglobin. They found that heat treatment (70−130 °C for 30 min) increased the antioxidant activity of whey while addition of the sulfhydryl blocker, iodoacetic acid (IAA), decreased it, indicating that sulfhydryl groups in the whey protein were involved. However, the authors also reported that not all sulfhydryls were blocked (total sulfhydryls decreased from 152 to 90 μM for skim milk) and that IAA can react slowly with lysine, histidine, and methionine (Means and Feeney, 1971), amino acids that could potentially influence lipid oxidation reactions (Decker, 1998).

Allen and Wrieden (1981a,b) studied the influence of whey proteins on lipid oxidation in a lysophosphatidylcholine-stabilized, copper-catalyzed trilinolein emulsion system. Proteins were added at levels similar to that found in milk. Whey was found to be slightly antioxidative, and of the whey components, α-lactalbumin (α-la) was more active than β-lactoglobulin (β-lg). β-Lg's smaller antioxidative effect was attributed to its buried sulfhydryl group, and it was unexplained as to why α-la had a stronger effect, given that it contains no free sulfhydryl groups. Lactoferrin (LF) was found to be slightly antioxidative, probably due to its ability to bind iron.

Donnelly et al. (1998) found continuous-phase whey protein isolate (WPI) in Tween 20-stabilized menhaden oil emulsions to be prooxidative. However, when an excess of Tween 20 was added to the aqueous phase in combination with WPI, an antioxidative effect was observed. The ability of surfactants to alter protein conformation, increasing the accessibility of free radical scavenging amino acids (cysteine and tyrosine) or iron chelating amino acids, was speculated to be the reason for increased antioxidant activity.

Ostdal et al. (1996) found that β-lactoglobulin is able to inactivate prooxidative heme proteins (ferrylmyoglobin). This inactivation leads to the formation of dityrosine in β-lactoglobulin, the cross-linked oxidation product of two tyrosines, thus suggesting that whey proteins may be able to scavenge free radicals.

While these studies yielded interesting results concerning the antioxidant activity of whey, the exact antioxidant mechanisms are still unclear. Also, it is difficult to predict how whey would impact oxidation in many food systems since most of these studies did not use prooxidants typical of a food emulsion nor did they use lipids with the same physical properties of food oil-in-water emulsions (e.g., Taylor and Richardson, hemoglobin and methyl linoleate; Allen and Wrieden, copper and trilinolein; Ostdal et al., a nonlipid system and ferrylmyoglobin). In addition, many of the whey proteins used were dried; therefore, their processing histories were unknown (Allen and Wrieden, 1982a,b; Ostdal et al., 1996; Donnelly et al., 1998), making data interpre-

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tation difficult since heat processing of whey can greatly affect the antioxidant activity of whey proteins (Taylor and Richardson, 1980).

The objective of this research is to better understand the antioxidant mechanism(s) of whey proteins in a salmon oil-in-water emulsion. By understanding the mechanisms by which whey inhibits lipid oxidation, technologies could be developed to maximize antioxidant activity, thus increasing the utilization of whey as a food antioxidant.

MATERIALS AND METHODS

Materials. Salmon fillets and pasteurized skim milk were purchased from a local grocer. Raw milk was obtained from a local dairy. Porphyridium cruentum β-phycoerythrin (β-PE), 2-thiobarbituric acid (TBA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-ethylmaleimide (NEM), and thimerosal were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane)dichloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Ferrous sulfate was obtained from MCB Manufacturing Chemist Inc. (Cincinnati, OH). Trichloroacetic acid was obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were reagent or HPLC grade and were obtained from Sigma Chemical Co. or Fisher Scientific.

Methods. *Preparation of Salmon Oil.* To obtain fresh salmon oil, salmon fillets were hand chopped into small pieces and minced in a food processor. The mince was then centrifuged at 10 000 rpm for 20 min, and the resulting lipid was decanted and stored at −80 °C until use. The resulting salmon oil consisted of (99.5 ± 0.2)% triacylglycerol (Mei et al., 1998). The levels of oxidation products initially in the oil was 0.32 mmol of lipid peroxide/kg of oil (as determined by a modification of the method of Shantha and Decker, 1994) and 0.04 mmol of TBA reactive substances (as determined by the method of McDonald and Hultin, 1987).

Preparation of Emulsion. A Tween 20-stabilized salmon oil-in-water emulsion was used in the lipid oxidation studies. A coarse emulsion consisting of 40% (w/w) salmon oil, 4% Tween 20, and 56% 50 mM phosphate buffer (pH 7) was made by homogenizing lipid and aqueous phases for 2 min using a Brinkman PT 10/35 Polytron (Westbury, NY) at a speed setting of 7. The coarse emulsion was then sonicated with a Braun-Sonic U (B. Braun Biotech, Allentown, PA) at 4 °C for 3 min at maximum power and 0.5 duty cycle. The final emulsion droplet size was 0.91 μm, as determined by laser light scattering (Mancuso et al., 1999). The emulsion was diluted with pasteurized or raw whey, whey fractions, and/or buffer to a final lipid concentration of 10%. Thimerosal (1 mM) was added to prevent bacterial spoilage.

For iron binding studies (ζ-potential measurements), a BSA-stabilized hexadecane-in-water emulsion was used in all experiments. A coarse emulsion consisting of 10 g of hexadecane, 2 g of BSA, and 488 g of water was made using a Brinkman PT 10/35 Polytron at a speed setting of 7 for 2 min. The coarse emulsion was passed six times through a APV−Gaulin model mini-lab 8.30H high-pressure valve homogenizer (Wilmington, MA) at 5000 psi.

Preparation of Whey Components. Acid whey was made using raw or pasteurized skim milk by adjusting the pH to 4.6, removal of casein by centrifugation (1000 × g, 10 min), readjusting to pH 7.0, and recentrifugation (1000 × g, 10 min). A high molecular weight (HMW) fraction of whey was isolated by dialysis against 50 mM phosphate buffer (pH 7.0; 100:1) with 3500 molecular weight cutoff dialysis tubing (Spectrum, Gardena, CA) a total of three times after 3, 6, and 12 h of constant stirring at 5 °C. The protein content of the HMW fraction of whey (14.0 mg of protein/mL) was measured using the Biuret method (Chang, 1994). The HMW fraction from raw whey was stored at −80 °C and thawed with tap water immediately prior to use. The HMW fraction of raw whey was exposed to heat treatments of 55, 60, 65, 70, 80, and 90 °C for 15 min in a water bath. Heat time included the time to reach desired temperature.

The "reactive" sulfhydryl content of the whey and continuous phase whey proteins was measured using a modification of Ellman's assay (Robyt and White, 1987). "Reactive" sulfhydryls were measured with the protein in its native conformation or after each individual heat treatment. Separation of the emulsion droplets from the continuous phase was accomplished by adding 1 mL of butanol to 6 mL of emulsion and centrifuging (1000 × g, 15 min). The lower aqueuous phase (2 mL) of the emulsion was collected, of which 2 mL was added to 0.1 mL of buffer (1 M Tris and 1 M phosphate, pH 8.1) and 0.5 mL of 2 mM 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB, Ellman's reagent). The mixture was allowed to react for 30 min at room temperature and centrifuged at 1000 × g for 5 min. The absorbance of the solution was determined at 412 nm. This value was corrected for scattering by also measuring the turbidity of the solution at 540 nm and using the formula $Abs_{412\ nm} - Abs_{540\ nm}$ (Taylor and Richardson, 1980). Concentrations of sulfhydryls were determined from a standard curve prepared using cysteine.

N-Ethylmaleimide (NEM) was used to block protein sulfhydryls (Friedman, 1973). The protein solutions (14 mg/mL) and NEM were allowed to react for 15 min at 25 °C. The level of NEM used was 3.45 mmol per gram of protein (final concentration = 50 mM). Excess NEM was removed by dialysis against 50 mM phosphate buffer (pH 7.0; 100:1) with 3500 molecular weight cutoff dialysis tubing a total of three times after 3, 6, and 12 h of constant stirring at 5 °C.

Lipid Oxidation Measurements. Emulsions (6 mL) were placed in tightly sealed screw cap test tubes (13 × 125 mm) and allowed to autooxidize at 20 °C for up to 21 days. Controls contained the salmon oil emulsion with or without NEM (50 mM). Lipid peroxides were measured (Shantha and Decker, 1994) by mixing the emulsion (0.3 mL) with 1.5 mL of isooctane/2-propanol (3:1, v/v), by vortexing (10 s, 3 times), and isolation of the organic solvent phase by centrifugation at 1000 × g for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/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 mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solutions was measured at 510 nm 20 min after addition of the iron. Peroxide concentrations were determined using a standard curve made from hydrogen peroxide.

Thiobarbituric acid reactive substances (TBARS; McDonald and Hultin, 1987) were determined by mixing 0.1 mL of emulsion with 0.9 mL of water and 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. The absorbance was measured at 532 nm. Concentrations of TBARS were determined from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

The scavenging of free radicals by whey was tested by its ability to prevent the peroxyl radical (originating from AAPH) initiated decay of porphyridium cruentum β-phycoerythrin (β-PE) fluorescence according to Cao et al. (1996). Whey (heated and unheated [140−0.7 μg/mL], with and without NEM) and β-PE (1.5 μg/mL) were mixed into 50 mM phosphate buffer (pH 7.0). Fluorescence decay was monitored at 22 °C after addition of AAPH (6.5 mM) for 15 min using excitation and emission wavelengths of 545 and 575 nm, respectively, on a Hitachi F-2000 Fluorometer (Tokyo, Japan). Fluorescence decay was calculated as the area between the curve area of β-PE alone and the sample in question. Percent inhibition was calculated as [1− (curve area of whey + AAPH + β-PE)/(curve area of AAPH + β-PE)] × 100. The HMW fraction which had been heated to >80 °C could not be analyzed due to light scattering by protein aggregates.

Measurement of Iron Chelation. ζ-Potential is a measure of the emulsion droplet surface charge and was measured using an adaptation of the method of Mei et al. (1998). ζ-Potential measurements were carried out to establish the

ability of whey proteins to chelate iron. By measuring the reduction in negative charge of oil droplets to which iron is absorbed after the addition of whey protein to the aqueous phase, it is possible to obtain information about iron chelation. For this reason, BSA was used as an emulsifier (rather than Tween 20) because it was negatively charged and therefore bound positively charged iron ions. Hexadecane was used as a nonoxidizable lipid to avoid potential alterations in interfacial properties by lipid oxidation products, which could be produced in emulsions containing polyunsaturated fatty acids. Iron (1.0−1000 μM $FeSO_4$, final concentrated) was added to the BSA-stabilized 2% hexadecane emulsion followed by the HMW fraction of whey (0.0−14.0 μg/mL, final protein concentration) or EDTA (20 μM final concentration), and then the emulsion was diluted to 0.1% lipid with double-distilled water. The diluted emulsion was injected into a ZEM5002 Zetamaster unit (Malvern Instruments, Ltd., Worcstershire, U.K.), and the ζ-potential (mV) was measured. Data represents averages of three readings of three separate injections. Performance of the instrument was monitored by using a DTS5050 standard colloidal suspension obtained from Malvern Instruments.

Statistics. All experiments were performed on triplicate samples. Differences between means were determined with the least-squares means procedure at $P < 0.05$ (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Concentration Effect of the Antioxidant Activity of Whey Protein. The antioxidant activity of the HMW fraction of whey from pasteurized milk was found to increase with concentration, as determined by its ability to inhibit TBARS and lipid peroxide formation (Figure 1a,b, respectively). The HMW fraction of whey (700, 1400, 4900, and 9800 μg of protein/mL) inhibited 17%, 73%, 95%, and 96% of TBARS formation and 29%, 70%, 78%, and 90% of lipid peroxide formation, respectively, in comparison to the control after 15 days of storage. The HMW fraction of whey contains proteins and phospholipid membranes. Research has shown that the phospholipids in the HMW fraction do not impact lipid oxidation in the salmon oil emulsions, indicating that the proteins are primarily responsible for the antioxidant activity (data not shown).

Effect of Sulfhydryls on the Antioxidant Activity of the HMW Fraction of Whey. Sulfhydryls are known free radical scavengers (Wayner et al., 1987; Darkwa et al., 1998). *N*-Ethylmaleimide binds strongly to sulfhydryl groups, forming a stable thiol adduct that is unavailable for further redox reactions (Friedman, 1973). Experiments were run to determine the level of NEM (5−200 mM) required to block a maximum of thiol groups, with excess NEM being removed by dialysis. A level of 50 mM NEM was chosen, as higher levels did not result in additional loss of sulfhydryl groups (Figure 2). Although this NEM concentration is in excess compared to the concentration of sulfhydryl groups present in the whey protein (30−35 μM), not all of the sulfhydryl groups could be blocked, with 14% of the reactive sulfhydryls remaining.

Figure 3 shows the oxidation of salmon oil emulsions containing HMW fraction (700 μg of protein/mL) and NEM-blocked HMW fraction (700 μg of protein/mL) of whey from pasteurized milk. NEM by itself had no significant effect on oxidation. The HMW fraction decreased TBARS formation 79% after 7 days of storage. The NEM-blocked whey was also able to inhibit TBARS formation; its effectiveness was reduced 60% compared to the HMW fraction alone at 7 days of storage. After 10 days, the antioxidant activity of the HMW and NEM-blocked HMW fractions disappeared, which suggests the depletion of sulfhydryl groups or other radical scavenging components of the HMW fraction.

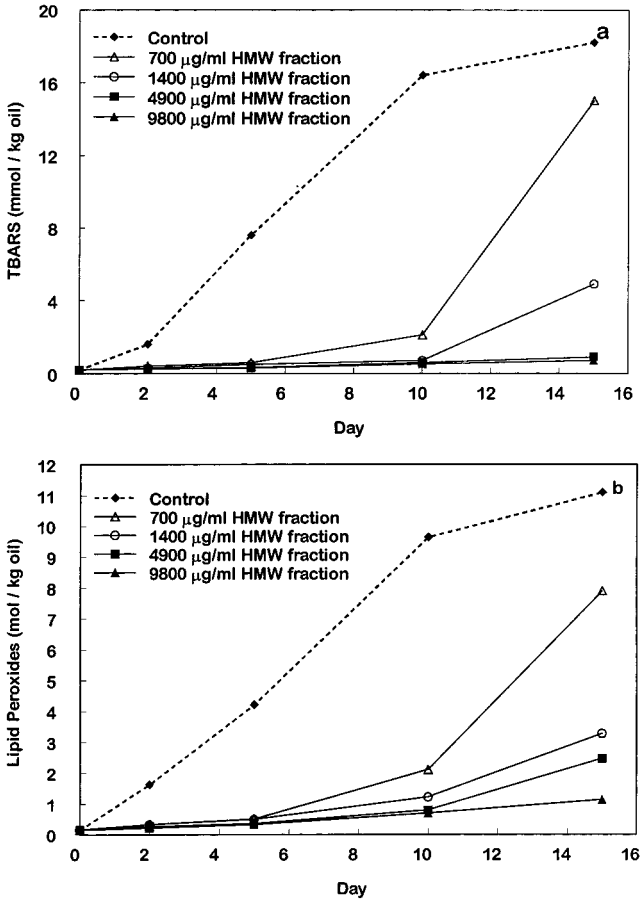

Figure 1. Effect of the HMW fraction of pasteurized whey concentration (mg/mL) on the formation of thiobarbituric acid reactive substances (a, TBARS) and lipid peroxides (b). Data represents means ± standard deviations. Some error bars lie within data points.

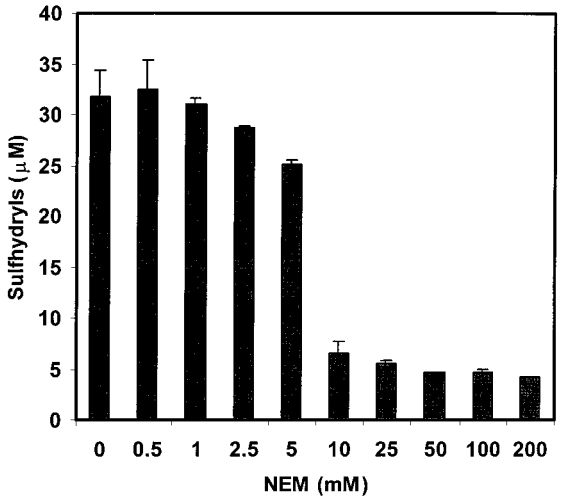

Figure 2. Influence of NEM concentration on the reactive sulfhydryl concentration of the HMW fraction of pasteurized whey. Data represents means ± standard deviations. Some error bars lie within data points.

If sulfhydryls are the sole antioxidant component of the HMW fraction of whey, once sulfhydryls are depleted, a rapid rise in the oxidation rates would be expected. HMW fraction of whey heated to 80 °C for 15 min (9800 μg of protein/mL) was added to 5% salmon

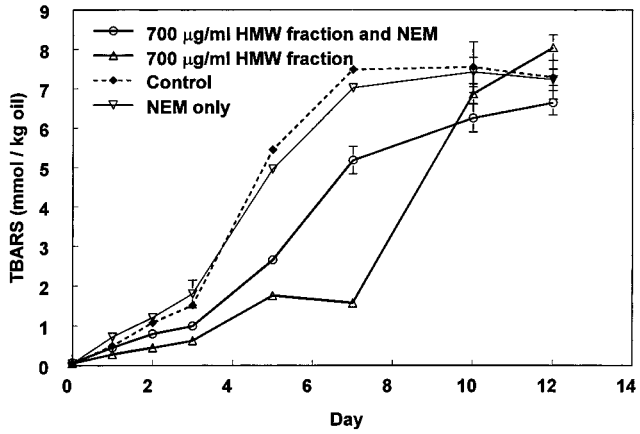

Figure 3. Influence of sulfhydryl blocking by *N*-ethylmaleimide (NEM) on the antioxidant activity of pasteurized HMW fraction whey (0.7 mg/mL) in a Tween 20-stabilized 5% salmon oil emulsion. Data represents means ± standard deviations. Some error bars lie within data points.

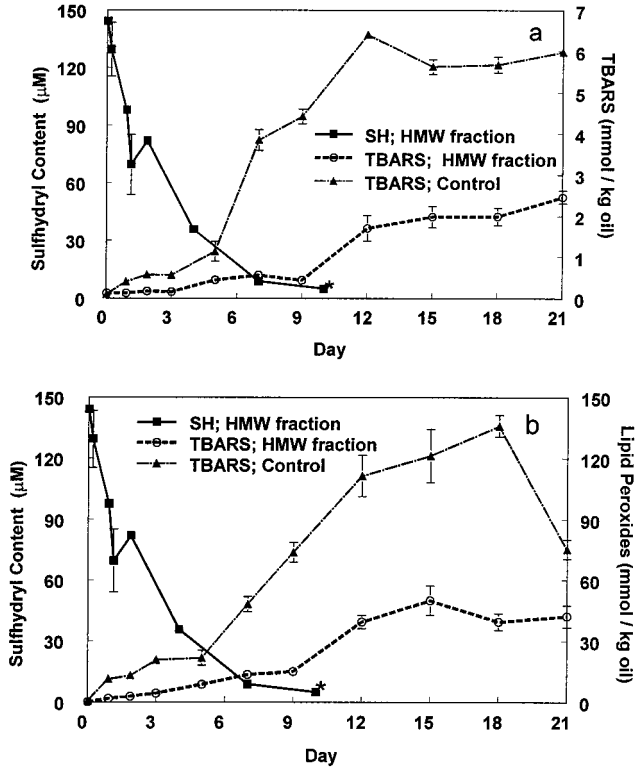

Figure 4. Effect of 80 °C heated HMW fraction whey protein (9800 μg/mL) on the formation of thiobarbituric acid reactive substances (a, TBARS) and lipid peroxides (b) and decline of sulfhydryls in Tween 20-stabilized 5% salmon oil emulsions. *Sulfhydryl concentration below detection limit (5 μM). Data represents means ± standard deviations. Some error bars lie within data points.

oil emulsions, and the formation of oxidation products (TBARS and lipid peroxides) and loss of sulfhydryls were measured (Figure 4). The HMW fraction of whey was added at 70% of the assay volume to provide protein concentrations high enough to measure changes in sulfhydryl concentrations. The HMW fraction of whey exerts a strong antioxidant effect, with 59% and 43% of oxidation inhibited after 21 days (TBARS and lipid peroxides, respectively). The sulfhydryl content of the HMW fraction proteins was depleted rapidly, with 97% of the sulfhydryls lost after 10 days. Between 10 and 21 days of storage, the HMW fraction whey still exerted

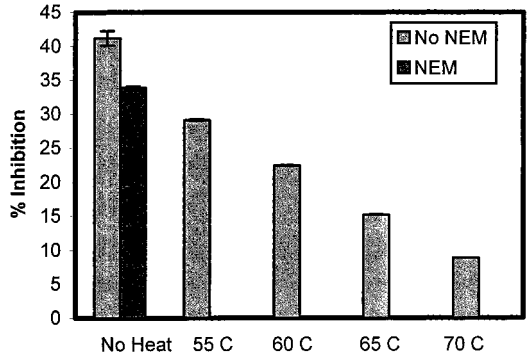

Figure 5. Influence heat treatment and sulfhydryl blocking by *N*-ethylmaleimide (NEM) on the inhibition of β-phycoerythrin decay as initiated by the free radical generator 2,2′-azobis-(2-amidinopropane) dichloride (AAPH) by raw HMW fraction whey. See Materials and Methods section for definition of % Inhibition. Data represents means ± standard deviations. Some error bars lie within data points.

an antioxidative effect even though the majority of the sulfhydryls were oxidized.

These results suggest that sulfhydryl groups are only partially responsible for the antioxidant activity of whey in salmon oil emulsions, suggesting that other antioxidant mechanisms such as free radical scavenging amino acids (e.g., tyrosine) and/or transition metal chelation by proteins such as lactoferrin and BSA could also be involved. This agrees with the results of Taylor and Richardson (1980), who observed that the antioxidant activity of whey was only partially lost by the sulfhydryl blocker, iodoacetic acid.

Free Radical Scavenging by the HMW Fraction of Whey. The ability of HMW fraction from raw whey to scavenge free radicals was measured in a nonlipid system by determining the ability of the HMW fraction proteins to inhibit AAPH-initiated fluorescent decay of β-PE as an indication of free radical scavenging activity (Cao et al., 1996). Different amounts of whey (0.7−140 μg protein/mL) were evaluated (data not shown), and a level that showed intermediate scavenging of peroxyl radicals (35 μg of protein/mL, HMW fraction) was chosen for further experiments. For the unheated HMW fraction, 42% inhibition of β-PE decay was observed, indicating that the HMW fraction of whey was able to scavenge peroxyl radicals (Figure 5). When sulfhydryls were blocked with NEM, scavenging of peroxyl radicals decreased approximately 20% compared to the HMW fraction without NEM. NEM alone did not scavenge peroxyl radical (data not shown). The inability of NEM (which blocks >85% of sulfhydryls in the HMW fraction) to completely inhibit peroxyl radical scavenging suggests that other protein components are also active free radical scavengers, such as tyrosine (Ostdal et al., 1996).

When heated, the ability of HMW fraction whey to scavenge peroxyl radicals decreases, from 42% (no heat treatment) to 9% (70 °C heat treatment). The influence of higher heating temperatures on peroxyl radical scavenging activity could not be determined due to protein aggregation, which interfered with the assay. The decline in radical scavenging with increasing heat treatment is possibly due to protein aggregation, which may prevent heat-exposed hydrophobic amino acids (e.g., tyrosine) from interacting with the water-soluble peroxyl radicals.

Ability of HMW Fraction of Whey To Remove Iron from Emulsion Droplet Surfaces. Iron is a

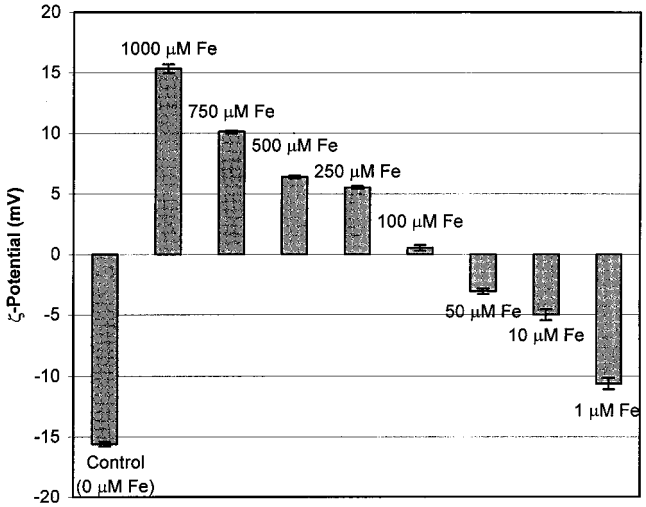

Figure 6. Effect of iron concentration on the ζ-potential of a BSA-stabilized 0.1% hexadecane emulsion. Data represents means ± standard deviations. Some error bars lie within data points.

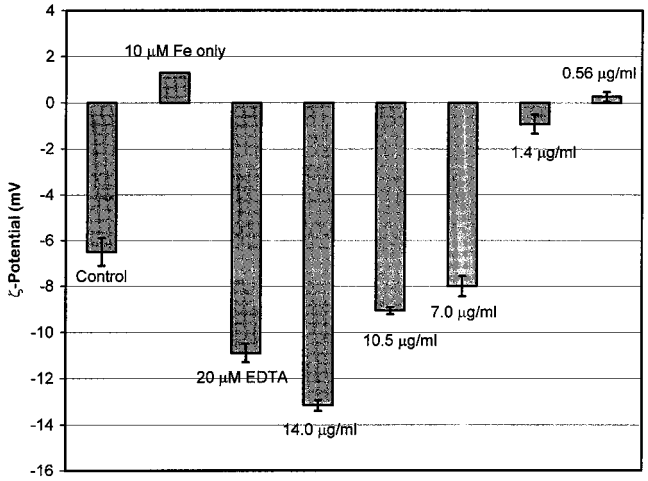

Figure 7. Effect of whey concentration on the ζ-potential of a BSA-stabilized 0.1% hexadecane emulsion. All samples contain 10 μM $FeSO_4$ except for the control. Data represents means ± standard deviations. Some error bars lie within data points.

strong prooxidant, especially when associated with negatively charged emulsion droplet surfaces (Mancuso et al., 1999; Mei et al., 1998). If whey protein is able to remove iron from emulsion droplet surfaces, whey could inhibit iron-induced oxidation. The ζ-potential of a BSA-stabilized hexadecane emulsion (pH 7.0) was found to be −15.6 mV (Figure 6). Ferrous sulfate (1−1000 μM) increased the charge of the emulsion droplets from −10.6 to 15.3 mV, indicating that iron was associated with the emulsion droplet surface. Higher iron concentrations (100−1000 μM) caused a charge reversal, due to its multivalent nature. These findings agree well with the findings of other authors (Mei et al., 1999; Kippax et al., 1999), who found that multivalent cations such as iron and calcium are able to increase and sometimes reverse the charge of negatively charged emulsion droplets.

The HMW fraction of whey from raw milk (14.0−0.56 μg of protein/mL) was added to a BSA-stabilized hexadecane emulsion containing 10 μM $FeSO_4$ to determine if the HMW fraction could chelate and remove iron from the emulsion droplet surface (Figure 7). The no iron, no chelator control had a ζ-potential of −6.5 mV. Upon addition of 10 μM iron, the charge was increased to 1.3 mV. Addition of the HMW fraction of whey to emulsions containing iron resulted in a decrease in the ζ-potential. The effect was concentration dependent, with 14.0 μg of protein/mL of HMW fraction showing the greatest effect, decreasing the ζ-potential to −13.2 mV. For comparison, EDTA (20 μM) also removed iron from emulsion droplet surfaces, as indicated by a decrease in the ζ-potential to −10.9 mV. Both the HMW fraction of whey (7.0−14.0 μg of protein/mL) and EDTA decreased ζ-potentials to levels below no iron controls (−6.5 mV), an effect that is likely due to their ability to remove endogenous cations (iron and calcium) from the emulsion droplet surface. EDTA has been previously seen to decrease the ζ-potential of sodium dodecyl sulfate-stabilized emulsions to levels below no iron controls (Mei et al., 1998). These data indicate that the HMW fraction of whey is able to chelate and remove iron away from the surface of negatively charged BSA-stabilized emulsion droplets surfaces. Lactoferrin (Gutteridge et al., 1981) and BSA (Meucci et al., 1991) have been reported to have iron chelating activity. It is unknown if other whey proteins are involved in the observed iron binding activity.

CONCLUSIONS

The antioxidant activity of the HMW fraction of whey is concentration dependent, with greater inhibition of oxidation occurring at higher concentrations. The mechanism of the antioxidant activity of HMW fraction whey in a salmon oil emulsion is dependent on sulfhydryl availability, with substantial loss of antioxidant activity occurring when sulfhydryl groups are blocked. However, whey proteins retain significant antioxidant activity when the majority of their sulfhydryl groups are blocked by NEM or lost by oxidation. An additional antioxidant mechanism of whey proteins seems to be free radical scavenging by other amino acids, as shown by the ability of the HMW fraction of whey to inhibit peroxyl radical initiated β-PE decay after sulfhydryls are blocked by NEM. Another possible antioxidant mechanism involves the ability of whey proteins to chelate iron. While whey proteins seem to inhibit lipid oxidation by several different mechanisms, sulfhydryl groups seem to be the most important antioxidant component.

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