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Role of Continuous Phase Protein on the Oxidative Stability of Fish Oil-in-Water Emulsions

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Whey protein isolate (WPI), soy protein isolate (SPI), and sodium caseinate (CAS) can inhibit lipid oxidation when they produce a positive charge at the interface of emulsion droplets. However, when proteins are used to stabilize oil-in-water emulsions, only a fraction of them actually absorb to the emulsion droplets, with the rest remaining in the continuous phase. The impact of these continuous phase proteins on the oxidative stability of protein-stabilized emulsions is not well understood. WPI-stabilized menhaden oil-in-water emulsions were prepared by high-pressure homogenization. In some experiments WPI was removed from the continuous phase of the emulsions through repeated centrifugation and resuspension of the emulsion droplets (washed emulsion). Unwashed emulsions were more oxidatively stable than washed emulsions at pH 7.0, suggesting that continuous phase proteins were antioxidative. The oxidative stability of emulsions containing different kinds of protein in the continuous phase decreased in the order SPI > CAS > WPI, as determined by both hydroperoxide and headspace propanal formation. Iron-binding studies showed that the chelating ability of the proteins decreased in the order CAS > SPI > WPI. The free sulfhydryls of both WPI and SPI were involved in their antioxidant activity. This research shows that continuous phase proteins could be an effective means of protecting $\omega-3$ fatty acids from oxidative deterioration.

KEYWORDS: **$\omega-3$ fatty acids; antioxdiants; proteins; lipid oxidation; emulsions**

INTRODUCTION

Omega−3 fatty acids are susceptible to lipid oxidation, a reaction that leads to the generation of unacceptable flavors, thus limiting the incorporation of these valuable fatty acids into foods (*1*). To utilize nutritionally beneficial omega−3 fatty acids in foods, their oxidative stability must be increased. Protein, both at the interface of emulsion droplets and in the continuous phase of oil-in-water emulsions, can inhibit lipid oxidation (*2*−*4*). Because proteins can be used as emulsifiers to produce physically stable oil-in-water emulsions while also inhibiting lipid oxidation, they could be useful multifunctional ingredients that could be used to aid the incorporation of oxidatively unstable lipids into foods.

β-Lactoglobulin, α-lactoalbumin, and serum albumin are the predominant proteins in whey (*5*). Both β-lactoglobulin and serum albumin contain one free sulfhydryl group. As with other proteins, whey proteins at the interface of emulsion droplets will inhibit lipid oxidation by producing a cationic surface charge that repels transitions metals when the pH is less than the p*I* of the proteins. In a comparison of the lipid oxidation rates in salmon oil-in-water emulsions where whey protein isolate (WPI), sweet whey, β-lactoglobulin, and α-lactalbumin were at the interface of the emulsion droplets at pH 3, the oxidative stability was found to be in the order β-lactoglobulin > sweet whey > WPI > α-lactalbumin (*4*). Whey proteins can also inhibit lipid oxidation when they are in the continuous phase of oil-in-water emulsions. Tong et al. (*3*) reported that the ability of continuous phase whey proteins to inhibit lipid oxidation was due to a combination of free radical scavenging by free sulfhydryl groups and chelation of prooxidant metals.

Sodium caseinate is an excellent emulsifying agent (*6*), and it is composed of four principle proteins: α_{s1}-, α_{s2}-, β-, and κ-casein (*5*). Caseins are disordered and substantially hydrophobic, which assists their rapid absorption during emulsification. This condition in turn leads to the rapid establishment of a thick stabilizing layer that protects newly formed droplets against flocculation and coalescence (*7*). Casein and casein-derived peptides are able to inhibit lipid oxidation by scavenging the free radical intermediates (*8*) and chelating prooxidant metals (*9*).

Soy proteins also form a stable oil-in-water emulsion. The major forms of soy proteins are the 7S and 11S globulins (*10*) with molecular masses of approximately 200,000 and 350,000 Da (*11*). Total and free sulfhydryl contents of soy protein isolate (SPI) are 8.0 and 5.0 μmol/g of protein respectively (*12*). SPI has been shown to be antioxidative in ground beef (*13*) and to inhibit lipid oxidation in phophatidylcholine liposomes (*14*). Soy proteins contain isoflavones, which are effective peroxyl radical scavengers (*15*).

By controlling the type, location, and concentration of protein in emulsions, the oxidative stability of oils high in omega−3 fatty acids could be increased (*4*, *11*, *16*). Casein, WPI, and SPI can all inhibit lipid oxidation when used as emulsifiers by producing cationic emulsion droplets when the pH is below their

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p*I* values (*11*). Very little is known about the ability of these proteins to inhibit lipid oxidation when they are in the continuous phase of oil-in-water emulsions containing high amounts of $\omega-3$ fatty acids. The objective of this research is to better understand the mechanisms by which continuous phase proteins inhibit lipid oxidation in protein-stabilized oil-in-water emulsions and to compare the impact of different protein types. Ultimately our goal is to use proteins to protect $\omega-3$ fatty acids from oxidation and to increase the application of $\omega-3$ fatty acids by the food industry.

MATERIALS AND METHODS

Materials. Commercial menhaden oil that was unstabilized, deodorized, refined, and bleached (eicosapentaenoic acid, 10−17%; docosahexenoic acid, 7−12%) was donated by Omega Protein (Reedville, VA). Menhaden oil was stored in the dark in 50 mL glass containers at −80 °C until used (<3 months). Whey protein isolate (WPI; Bipro; 97.6% protein), soy protein isolate (SPI; Alpha 5800; 86.0% protein), and sodium caseinate (CAS; Alanate 110; 96.9% protein) were donated by Davisco (LeSueur, MN), Central Soya (Fort Wayne, IN), and New Zealand Milk Protein (Lemoyne, PA), respectively. Protein concentrations are those listed by the manufacturer. *N*-Ethylmaleimide, barium chloride, sodium acetate, ferrous sulfate, nitrilotriacetic acid, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Dialysis bags (23 mm × 30 m; molecular weight cutoff of 3500) were obtained from Spectrum Co. (Cardena, CA). All other chemicals were of reagent or HPLC grade and were obtained from Sigma Chemical or Fisher Scientific.

Methods. *Preparation and Characterization of Oil-in-Water Emulsions.* A WPI-stabilized menhaden oil-in-water emulsion was used in all lipid oxidation studies. An aqueous emulsifier solution was made by dispersing 2.0% WPI in 10 mM sodium acetate and 10 mM imidazole buffer (pH 3 or 7) and stirred overnight at 5 °C to ensure complete dissolution. The pH of the emulsifier solution was adjusted with 0.1 M HCl and/or NaOH. A coarse emulsion consisting of 10% (w/w) menhaden oil and 90% emulsifier solution was made by homogenizing lipid and aqueous phases for 2 min using a two-speed hand-held bio homogenizer (Biospec Products Inc., Bartlesville, OK) at the highest speed setting. The size of the emulsion droplets was then further reduced by two passes through a two-stage high-pressure valve homogenizer (AVP Lab 1000, Albertslund, Denmark) at 2000 psi.

The particle size distribution of the emulsions was measured using a laser light scattering instrument (Coulter LS-230; Miami, FL). To prevent multiple scattering effects, the concentrated emulsions were diluted with acetate/imidazole buffer at the same pH as the emulsion prior to analysis so that the droplet concentration was <0.005 wt % (*17*). The droplet size distributions were checked periodically to monitor emulsion stability. Particle size in all emulsions ranged from 0.4 to 0.6 μm and did not change during the course of the studies.

Removal and Quantitation of Continuous Phase Proteins. To produce washed emulsions, ∼37 g of emulsion was placed in centrifuge tubes (25.5 mm × 102 mm) and centrifuged for 50 min at 36000*g* at 10 °C using an ultracentrifuge (Sorvall Superspeed RC2-S; Sorvall Instruments, Wilmington, DE). After centrifugation, ∼32 g of the continuous phase (lower layer) of the creamed emulsion was discarded and replaced by an equal amount of acetate/imidazole buffer and vortexed for 1 min. This process was repeated a total of three times. Lipid content of the final washed emulsion was determined by a using modified method of Bligh and Dyer (*18*), after which the emulsion was diluted to 5% lipid. The washing procedure did not alter the particle size distribution of the emulsion droplet.

Continuous phase from the first and third centrifugations was collected and recentrifuged at 325000*g* and 10 °C for 1 h using a Sorvall Ultra 80 centrifuge. The continuous phase of the first centrifugation represented the total continuous phase protein concentration after homogenization. The continuous phase from the third centrifugation represented the total continuous phase protein concentration after the washing procedure was completed. After centrifugation, the lower continuous phase layer was filtered using a Nylon Acrrodic 13 (0.2 μm) syringe filter (Gelman Science, Ann Arbor, MI). Protein concentration was measured according to the Lowry procedure (*19*). A standard curve was prepared for each protein studied using authentic WPI, SPI, or CAS.

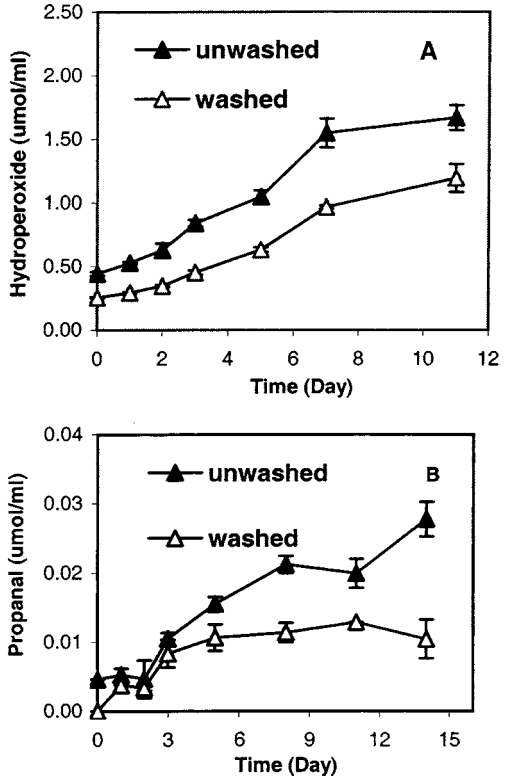

Figure 1. Formation of lipid hydroperoxides (**A**) and headspace propanal (**B**) in unwashed and washed 5% menhaden oil-in-water emulsions stabilized by 1.0% WPI at pH 3.0. Data points represent means ($n = 3$) ± standard deviations.

Lipid Oxidation Measurements. Emulsions (2 mL) were placed in capped test tubes (13 × 100 mm; Fisherbrand) and allowed to autoxidize in the dark at 20 °C for up to 15 days. Lipid hydroperoxides were measured according to the method of Shanta and Decker (*20*) by mixing the emulsion (0.3 mL) with 1.5 mL of isooctane/2-propanol (3:1, v/v), vortexing (10 s, three times), and isolating the organic solvent phase by centrifugation at 1000*g* for 2 min. The organic phase (top layer; 0.2 mL) 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 adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, the absorbance was measured at 510 nm using a UV−vis scanning spectrophotometer (Shimadzu UV-2101PC, Kyoto, Japan). Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide. Initial hydroperoxide concentrations in the menhaden oil were 1.3 μmol/mL of oil.

Headspace aldehydes were determined by placing 1 mL 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 headspace sampler Hewlett-Packard 19395A (*21*). 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 μm 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.

Blocking Protein Sulfhydryls. N-Ethylmalemide (NEM) was used to block protein sulfhydryls in some experiments (*22*). The protein solutions (25 mg/mL) and NEM were allowed to react for 15 min at 25 °C. The level of NEM used was 3.45 mmol/g of protein (*3*). Excess NEM was removed by dialysis at 5 °C against 100 parts of 10 mM sodium acetate and imidazole buffer (pH 7) with 3500 molecular weight cutoff dialysis tubing with buffer being changed at 6, 12, and 24 h (*3*).

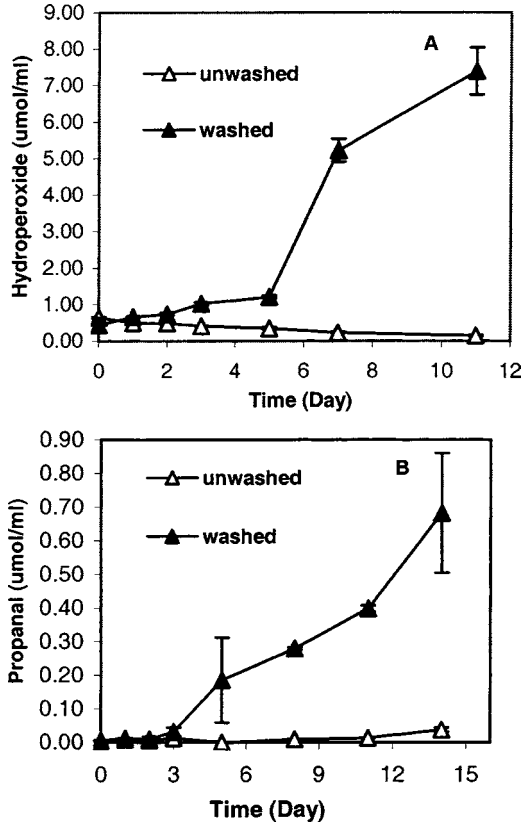

Figure 2. Formation of lipid hydroperoxides (**A**) and headspace propanal (**B**) in unwashed and washed 5% menhaden oil-in-water emulsions stabilized by 1.0% WPI at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

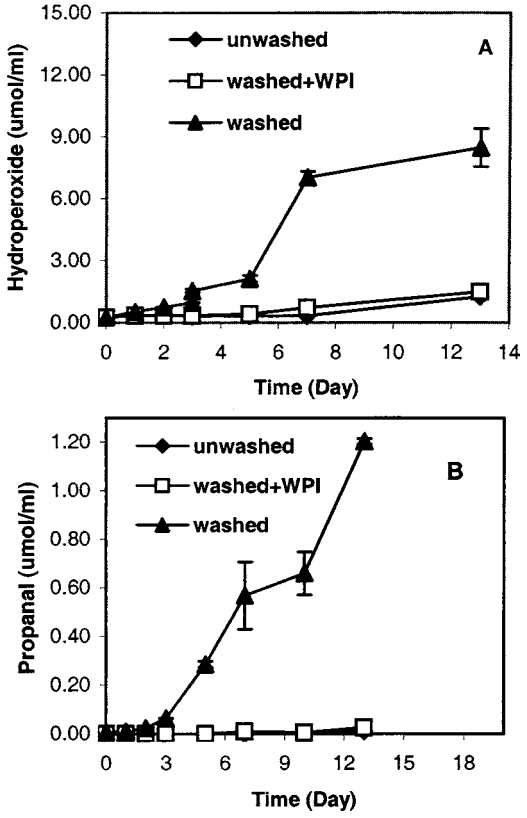

Figure 3. Effect of WPI added back to the continuous phase of washed 5% menhaden oil-in-water emulsions stabilized by 1.0% WPI on the formation of hydroperoxides (**A**) and propanal (**B**) at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

Iron Nitrilotriacetate−Protein-Binding Experiments. The ability of the proteins to chelate iron was determined using a modified method of Lin et al. (*23*). 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. Protein (10 mL) at different concentrations was placed inside a dialysis bag (3500 molecular weight cutoff) and was incubated in 1000 mL of the Fe-NTA buffer mixture for 24 h (*23*). After dialysis, the protein concentrations inside the dialysis bag were measured using the Lowry procedure (*19*).

A protein precipitating solution was prepared with hydroxylamine hydrocholoride (0.72 M), tricholoroacetic acid (0.61 M), and 100 mL of 12 N HCl. To measure protein-bound iron, protein samples from inside the dialysis bag (4 mL) were mixed with 2 mL of protein-precipitating solution and incubated overnight. Samples were then centrifuged at 1750*g* for 10 min. The resulting supernatants (1 mL), which contained 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 Ferrozine reagent. After 1 h, the absorbance was determined at 562 nm and iron concentration were determined with a standard curve prepared from $FeCl_3$ (*24*).

Statistical Analysis. Assays were measured in triplicate. Statistical analysis was performed using Student's *t* test (*25*).

RESULTS AND DISCUSSION

Effect of pH on the Oxidative Stability of Washed and Unwashed WPI-Stabilized Oil-in-Water Emulsions. WPI-stabilized emulsion droplets at pH values below the p*I* of the whey proteins (p*I* ~5.1) produce cationic emulsion droplets that can repel transition metals such as iron or copper (*4*). As expected, lipid hydroperoxide and propanal concentrations in the WPI-stabilized menhaden oil-in-water emulsion containing 0.5% WPI and 5% lipid were lower at pH 3.0 (**Figure 1**) compared to pH 7.0 (**Figure 2**). When proteins are used to stabilize menhaden oil-in-water emulsions, only a portion of them actually absorb to the emulsion droplets, with the rest remaining in the continuous phase. For instance, in a 5% menhaden oil-in-water emulsion stabilized with 0.5% WPI, 90% of the protein was at the emulsion droplet interface (**Table 1**). In the washed emulsions, where WPI was removed from continuous phase, the formation of lipid hydroperoxides and headspace propanal was slightly lower at pH 3.0 (**Figure 1**) and dramatically higher at pH 7.0 (**Figure 2**). The ability of the continuous phase proteins to inhibit lipid oxidation at pH 7.0 but not at pH 3.0 is likely due to the differences in the charge of the proteins. At pH 7.0 the continuous proteins would be anionic and thus would be able to chelate prooxidant metals. Alternately, washing could remove low molecular weight components in the emulsions that originated from the proteins used in this study. If these low molecular weight components

Table 1. Protein Concentration in the Continuous Phase of Washed and Unwashed Emulsion

% protein concn in emulsion	protein concn (μg/mL) in the continuous phase
unwashed	
0.25	122.7 ± 1.7
0.5	512.0 ± 12.0
1.0	1383.0 ± 32.0
1.5	2384.0 ± 17.0
washed	
0.25	4.0 ± 0.2
0.5%	5.0 ± 0.2
1.0%	11.0 ± 0.2
1.5%	13.0 ± 0.5

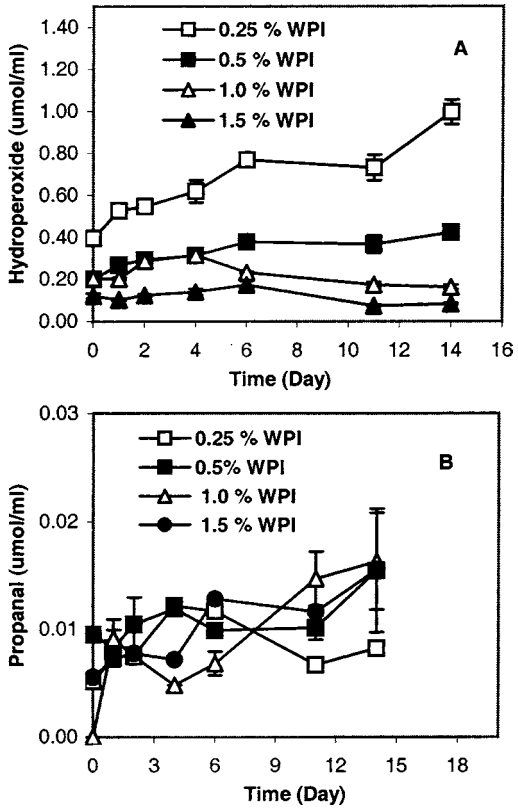

Figure 4. Formation of lipid hydroperoxides (**A**) and propanal (**B**) in unwashed 5% menhaden oil-in-water emulsions prepared with different WPI concentrations (0.25–1.5%) at pH 7. Data points represent means ($n = 3$) ± standard deviations.

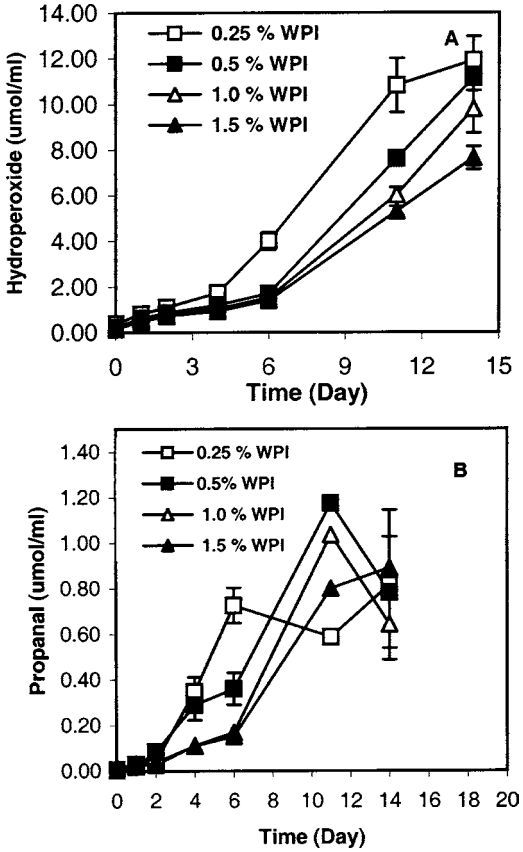

Figure 5. Formation of lipid hydroperoxide (**A**) and propanal (**B**) in washed 5% menhaden oil-in-water emulsions prepared with different WPI concentrations (0.25–1.5%) at pH 7. Data points represent means ($n = 3$) ± standard deviations.

had antioxidant activity, the washing procedure would increase oxidation rates. If this was the case, it would have to be antioxidants that were active at pH 7.0 but not pH 3.0 because washing did not affect oxidative stability at pH 3.0. Continuous phase WPI at pH 7.0 inhibited lipid oxidation as measured by both lipid hydroperoxides and headspace propanal, so all further studies were performed at this pH.

WPI was added back to washed emulsion at pH 7.0, and oxidation products were measured. Addition of theWPI to the continuous phase of washed emulsion at a concentration equal to the continuous phase WPI in the unwashed emulsion (prepared with 1.0% WPI) produced a level of oxidative stability similar to that of the unwashed emulsion (**Figure 3**), suggesting that differences in oxidative stability were due to the aqueous phase proteins and not the washing procedure.

Effect of WPI Concentration on Oxidative Stability of Washed and Unwashed Emulsions at pH 7.0. Previous work has shown that the surface of the emulsion droplets becomes saturated with protein when the WPI concentration exceeds 0.2% in an oil-in-water emulsion containing 5% lipid (*4*, *16*). Therefore, increasing protein concentrations from 0.25 to 1.5 wt % at a constant lipid concentration of 5% should result in an increase in continuous phase protein concentration. Increasing the concentration of WPI used to prepare the emulsion resulted in a reduction of emulsion droplet size (the mean particle diameter for unwashed emulsion containing 0.25% WPI was 0.59 μm compared to 0.46 μm for emulsion prepared with 1.5% WPI). The continuous phase protein concentration of unwashed emulsions decreased when decreasing amounts of WPI were used to prepare the emulsion. For instance, unwashed emulsion prepared with 1.5% WPI had >19 times more continuous phase protein than emulsion prepared with 0.25% WPI (**Table 1**). Washing the emulsions by repeated centrifugation and replacement of the continuous phase resulted in a decrease in continuous phase protein concentrations to <15 mg/mL (>97% reduction; **Table 1**).

Both lipid hydroperoxide and headspace propanal formations were much lower in the unwashed emulsions (**Figure 4**) compared to the washed emulsions (**Figure 5**), again indicating that the continuous phase proteins were antioxidative. Formation of lipid hydroperoxides was lower in unwashed emulsion prepared with high WPI concentrations (**Figure 4A**); however, no consistent differences in headspace propanal concentrations were observed among the different protein concentration treatments (**Figure 4B**). The ability of the continuous phase proteins to inhibit lipid hydroperoxide formation but not propanal formation could be due to the protein having multiple antioxidant mechanisms. Lipid hydroperoxide formation can be inhibited by free radical scavengers. The free sulfhydryls in whey proteins can act as free radical scavengers (*3*, *26*) and thus could be responsible for the inhibition of lipid hydroperoxide formation. Once lipid hydroperoxides are formed, they can be decomposed by prooxidant metals to form small molecular weight volatile compounds such as propanal. Whey protein can also act as a metal chelator (*3*) and thus could inhibit propanal formation. The inability of increasing continuous phase WPI concentration to further inhibit propanal formation suggests that even at the lowest protein concentrations (122.7 μg of protein/mL continuous phase in the emulsion prepared with 0.25% WPI) had more than enough continuous phase protein to inactivate the prooxidant metals in the emulsion. The washed emulsions made with low WPI concentrations (0.25%) had greater lipid hydroperoxide concentration after 6 and 11 days

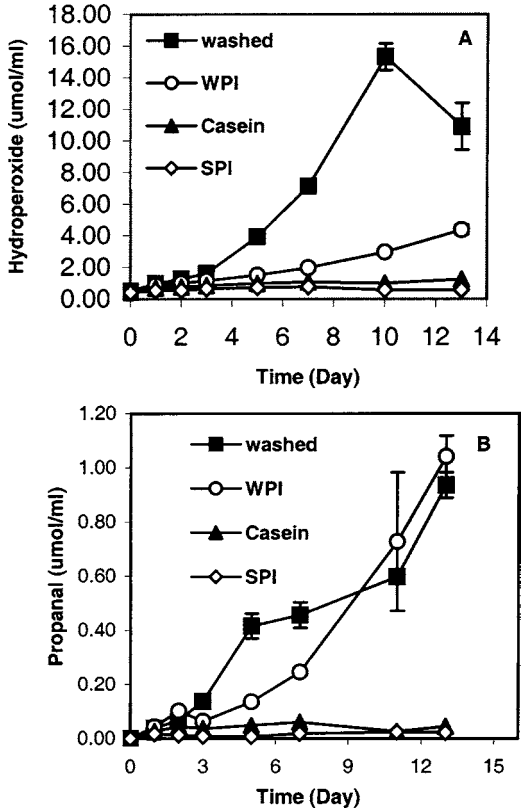

Figure 6. Influence of 0.05% continuous phase WPI, casein, and SPI on the formation of lipid hydroperoxides (**A**) and propanal (**B**) in washed 5% menhaden oil-in-water emulsions stabilized by WPI at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

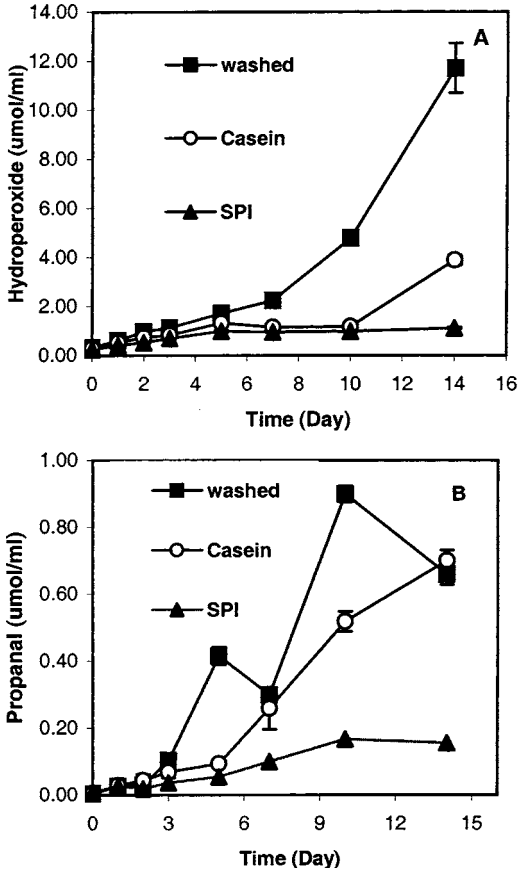

Figure 7. Influence of 0.01% continuous phase casein and SPI on the formation of lipid hydroperoxides (**A**) and propanal (**B**) in washed 5% menhaden oil-in-water emulsions stabilized by WPI at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

of storage and greater headspace propanal at 6 days than washed emulsions prepared with ≥0.5% WPI (**Figure 5**). This difference could again reflect differences in continuous phase protein concentrations because washed emulsions prepared with 1.5% WPI had >3.5-fold more continuous phase protein than washed emulsions made with 0.25% WPI.

Casein and soy protein isolate have both been reported to inhibit lipid oxidation (*8*, *9*, *27*). To determine if continuous phase CAS and SPI would inhibit lipid oxidation in oil-in-water emulsion, they were added to the washed WPI-stabilized emulsion at a concentration of 0.05%. Both CAS and SPI were more effective than WPI at inhibiting both lipid hydroperoxide and propanal formation (**Figure 6**). However, there were no significant differences in lipid hydroperoxides and propanal between the CAS and SPI treatments. Therefore, in the subsequent experiment CAS and SPI were added to WPI emulsions at a lower protein concentration (0.01%). At this protein concentration, lipid hydroperoxides (day 14) and headspace propanal (after day 7) were greater in emulsions with added CAS compared to SPI, suggesting that the antioxidant activity of SPI was better than that of CAS (**Figure 7**). Differences in the ability of these different proteins to inhibit lipid oxidation in the continuous phase of menhaden oil-in-water emulsions could be due to several factors such as differences in their ability to chelate prooxidant metals and/or scavenge free radicals. The antioxidant activity of the continuous phase proteins was quite different from the ability of these proteins to inhibit lipid oxidation when they were at the interface of corn oil-in-water emulsions at pH 3.0, where the oxidative stability of the emulsions was in the order CAS > WPI > SPI (*11*). It is possible that WPI at the emulsion droplet interface could be displaced by continuous phase CAS and SPI during the storage of the emulsions. Dalgleish and co-workers (*28*) found that whey proteins could displace interfacial casein in casein-stabilized soy oil-in-water emulsions, suggesting that the whey proteins were more surface active than casein. Because whey proteins are more surface active, it is unlikely that casein would displace WPI from the emulsion droplet interface in our emulsion system. Thus, the antioxidant effect of CAS is not likely to be due to its migration to the interface of the WPI-stabilized menhaden oil emulsion droplets. It is unknown if SPI would be more surface active than WPI and thus would displace WPI from the emulsion droplet interface. If this did occur and SPI became the major interfacial protein, it would be unlikely that an antioxidant effect would have been observed because it has been previously shown that SPI-stabilized oil-in-water emulsions are less oxidatively stable than WPI-stabilized oil-in-water emulsions (*11*). More research is needed to determine if interfacial exchange of individual proteins within these complex protein mixtures could be responsible for the observed differences in oxidative stability. Overall, these differences in oxidative stability highlight the notion that both the physical location and chemical properties of proteins in emulsion are important in their ability to alter lipid oxidation reactions.

Evaluation of the Antioxidant Mechanisms of Continuous Phase Proteins. Continuous phase proteins could be inhibiting lipid oxidation by chelating prooxidant metals or scavenging free radicals. In the negatively charged WPI-stabilized emulsions at pH 7.0, the transition metals are strong prooxidants that accelerate lipid oxidation (*29*). If chelators such as continuous phase proteins are able to bind iron and remove it from the emulsion droplet surface and/or from the lipid core, lipid

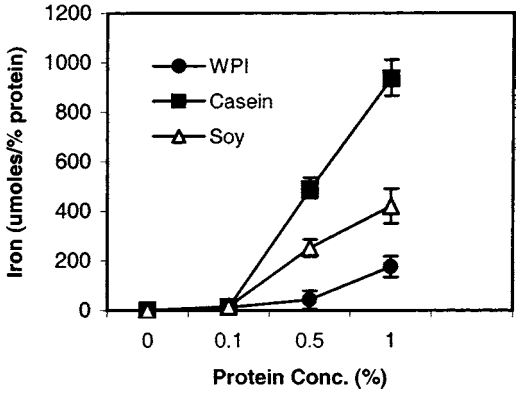

Figure 8. Ability of various concentrations (1–10 mg/mL) of WPI, casein, and SPI to bind iron after incubation for 24 h at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

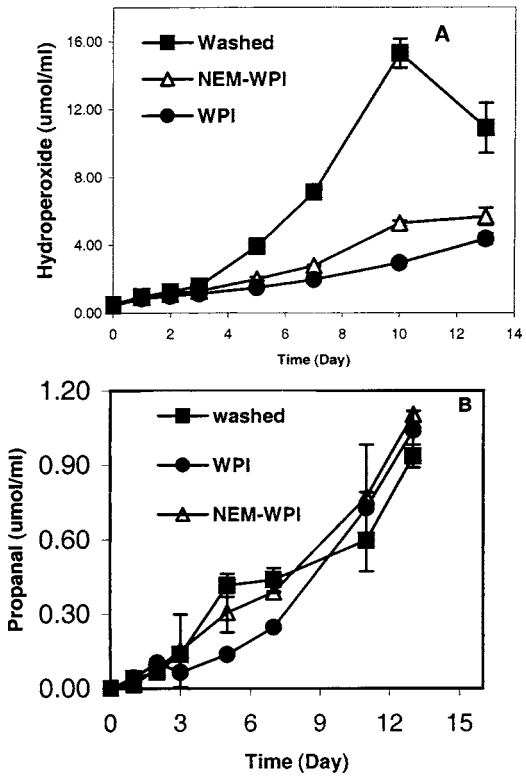

Figure 9. Influence of 0.05% continuous phase WPI and WPI whose free sulfhydryls were blocked with *N*-ethylmalemide (NEM–WPI) on the formation of lipid hydroperoxides (**A**) and propanal (**B**) in washed 5% menhaden oil-in-water emulsions stabilized by WPI at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

oxidation would be inhibited (*30*). Nitrilotriacetic acid (NTA) forms a complex with iron that keeps the iron soluble at pH 7.0, thereby making NTA-Fe complexes suitable for binding studies (*31*, *32*). Of the three proteins tested, CAS had the highest chelation activity, binding 2.2- and 5.3-fold more iron than SPI and WPI, respectively, at 10 mg of protein/mL (**Figure 8**). The greater chelating capacity of CAS is likely due to its phosphorylated serine residues, which are known to bind iron (*33*). The ability of WPI to chelate iron in oil-in-water emulsions is in agreement with the observations of Mei et al. (*34*) and Tong et al. (*3*). WPI was less effective at chelating iron than SPI.

The sulfhydryl groups of whey proteins have been shown to act as free radical scavengers that inhibit lipid oxidation when the whey proteins are in the continuous phase of Tween 20-

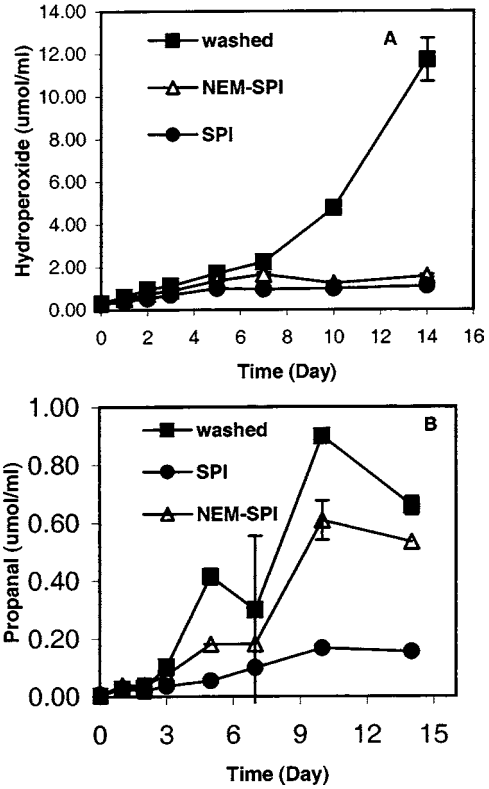

Figure 10. Influence of 0.01% continuous phase SPI and SPI whose free sulfhydryls were blocked with *N*-ethylmalemide (NEM–SPI) on the formation of lipid hydroperoxides (**A**) and propanal (**B**) in washed 5% menhaden oil-in-water emulsions stabilized by WPI at pH 7.0. Data points represent means ($n = 3$) ± standard deviations.

stabilized oil-in-water emulsons (*3*). Of the proteins tested, both WPI and SPI have free sulfhydryl groups. NEM binds strongly to SH groups, forming a stable thiol adduct that is not available for further redox reaction (*22*). Therefore, NEM-treated WPI (0.05%) and SPI (0.01%) were added to the continuous phase of the washed WPI-stabilized emulsion to determine the antioxidant role of sulfhydryls in continuous phase proteins. The ability of continuous phase WPI and SPI to inhibit lipid hydroperoxide and propanal formation was decreased but not eliminated by the NEM treatment (**Figures 9** and **10**). Decreased inhibition of lipid oxidation by NEM-treated WPI and SPI suggests that sulfhydryl groups are involved in the antioxidant activity of these proteins. Tong et al. (*3*) also found that whey protein sulfhydryls can inhibit lipid oxidation in salmon-oil-in-water emulsions stabilized with Tween 20. Hu et al. (*4*) found that blocking whey protein sulfhydryls with NEM did not inhibit lipid oxidation rates when the whey protein was at the emulsion droplet interface, again suggesting that the physical location of the proteins is an important factor in their ability to act as antioxidants.

Conclusions. Proteins in the continuous phase of menhaden oil-in-water emulsions can inhibit lipid oxidation at pH 7.0. Continuous phase proteins inhibit lipid oxidation in oil-in-water emulsion through a combination of free radical scavenging and metal chelation. Of the proteins tested, casein was the best iron chelator. The sulfhydryls of both WPI and SPI were involved in the ability of these continuous phase proteins to inhibit lipid oxidation. It is likely that other amino acids that can scavenge free radicals (e.g., tyrptophan, methionine, and tyrosine) could also be involved in the antioxidant activity of the continuous phase proteins. In addition, other antioxidants associated with the proteins could also act as antioxidants such as the isoflavone

associated with SPI, a factor that may help to explain why continuous phase SPI had the greatest antioxidant activity of all the proteins tested. Further research is needed to evaluate specific amino acids or other compounds that may be responsible for the antioxidant activity of these continuous phase proteins.

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