Antioxidant Activity of Whey in a Salmon Oil Emulsion

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ABSTRACT: The antioxidant capabilities of whey in a Tween 20™-stabilized salmon emulsion were investigated. Whey fractions inhibited formation of thiobarbituric acid reactive substances (TBARS) and lipid peroxides in a 10 % salmon oil emulsion in the order of whey > high-molecular-weight (HMW) fraction > low-molecular-weight fraction. Heating the HMW fraction exposed sulfhydryls, with optimum exposure at 80 °C. Heating the HMW fraction above 80 °C increased antioxidant activity. The HMW fraction of whey (80 °C) alone, α-tocopherol (40 ppm) alone, and their combination inhibited TBARS 59, 19 and 86%, respectively, at 21 d of storage. Sulfhydryls oxidized before α-tocopherol, suggesting that sulfhydryls are the primary antioxidant. Results indicate that whey proteins could be useful antioxidants in food emulsions.

Keywords: lipid oxidation, fish oil, α-tocopherol, whey, sulfhydryls

Introduction

THE HEALTH BENEFITS OF DIETARY OMEGA-3 (W-3) FATTY ACIDS include reduced susceptibility to malignant tumors, protection against heart disease, and improved brain and eye function in infants (Simopoulos 1991; Innis 1991). It is of great interest to food manufacturers to use ω-3 fatty acids as physiologically functional ingredients to improve the nutritional profile of food products. However, oxidation of ω-3 fatty acids is very rapid, thus causing rapid flavor deterioration, loss of nutrients, and formation of potentially toxic compounds (Kubow 1992; Frankel 1998). These problems have been major barriers to utilizing oils that contain high concentrations of ω-3 fatty acids in foods.

Oxidative stability of ω-3 fatty acids can be increased using free radical scavengers. Tertiary-butylhydroquinone (TBHQ, 0.02%) has been successfully employed to slow oxidation of menhaden oil for up to 40 days, compared to approximately 3 d for the control (Farkas and others 1997). Alpha-Tocopherol and butylated hydroxytoluene (BHT) alone or in combination increased the oxidative stability of octadecatetraenoic acid (ODTA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), inhibiting malonaldehye (MA) formation upon addition of Fenton's reagent (Ogata and others 1996). Alpha-Tocopherol inhibited MA formation from ODTA and DHA 67 and 28 % respectively, but had no activity toward EPA. BHT inhibited MA formation from ODTA, DHA, and EPA 80, 58 and 93 %, respectively (Ogata and others 1996). A combination of ascorbic acid (200 ppm) solubilized in lecithin reverse micelles and δ-tocopherol (4000 ppm) was also tested for its ability to decrease autoxidation of sardine oil, with the carbonyl value of the control reaching 45 at day 36 and the carbonyl value of the antioxidant-treated sample reaching 1.0 during the same period. (Han and others 1997).

If ω-3 fatty acids are to be successfully incorporated into processed foods, it will most likely be in the form of a lipid dispersion. While lipid-soluble free radical scavengers may be effective in minimizing oxidation for a limited amount of time in bulk oils, their effectiveness seems to be dependent on fatty acid type and they are not likely to be effective for the shelf-life required for most processed foods. In addition, the effectiveness of lipid-soluble free radical scavengers in oil-in-water emulsions can be different if they partition into both the continuous phase and emulsion droplet (Huang and others 1997). Therefore, if ω-3 fatty acid oils are to be successfully utilized in food emulsions, additional antioxidant hurdles must be utilized, such as continuous phase metal chelators and free radical scavengers.

Proteins are well known mediators of lipid oxidation. A number of antioxidative proteins and protein products exist, including soy and yeast protein hydrolysates (Bishov and Henick 1972 and 1975), gelatin (Kawashima 1979), and blood plasma proteins (Wayner and others 1987; Halliwell 1988). Mechanisms of the antioxidative effects of proteins include chelation of prooxidant transition metals by proteins such as lactoferrin (Gutteridge and others 1981) and serum albumin (Meucci and others 1991) and free radical scavenging by amino acid residues such as cysteine and tyrosine (Wayner and others 1987; Ostdal and others 1996).

Taylor and Richardson (1980) explored the antioxidant activity of heated skim milk in a methyl linoleate emulsion catalyzed by hemoglobin. They found that heat treatment (70 to 130 °C in a silicone oil bath, up to 30 min) increased the antioxidant activity of skim milk, an observation that was partially attributed to exposure of proteinaceous sulfhydryl groups. Heat treatment resulted in an increase in reactive sulfhydryls (48 mM sulfhydryls with no heat to 71 mM sulfhydryls after a 130 °C, 30 min heat treatment), but a decline in total sulfhydryls (146 mM sulfhydryls with no heat to 39 mM sulfhydryls after a 130 °C, 30 min heat treatment). Milk fractions were also evaluated, with both whey and casein being antioxidative, although casein showed much greater activity. The activity of casein was hypothesized to be mostly due to a physical binding of prooxidants, while the activity of whey was thought to be chemical in nature. While this study yielded interesting results concerning the antioxidant capabilities of skim milk and whey, it is difficult to predict how milk could impact oxidation in food systems since the research of Taylor and Richardson did not use prooxidants typical of a food emulsion (for example, hemoglobin), nor did they use lipids (methyl linoleate dispersion with Tween 80™) with the same physical properties of food oil-in-water emulsions.

Since ω-3 fatty acids added to foods are most likely to be in the form of lipid dispersions, we chose to explore the antioxidant capabilities of whey proteins in the continuous phase of a Tween 20™-stabilized salmon oil-in-water emulsion. Antioxidant interactions between whey proteins and α-tocopherol were explored as well as the potential of thermal processing, which could improve the antioxidant activity of whey. This research will hopefully encourage the use of whey protein in foods to retard lipid oxidation.

Materials and Methods

Materials

Salmon fillets and skim milk were purchased from a local grocer. Raw milk was obtained from a local dairy. 2-Thiobarbituric acid (TBA), polyoxyethylene sorbitan monolaurate (Tween 20™, ICI Americas Inc. Wilmington, Del., U.S.A.), 5,5'dithiobis[2-nitrobenzoic acid] (DTNB), 1,1,3,3-tetraethoxypropane, and thimerosal were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trichloroacetic acid was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). 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 for 3 min 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 and others 1998). The levels of oxidation products in the oil was 0.32 mmol lipid peroxides/kg oil (as determined by the method of Shantha and Decker 1994) and 0.04 mmol TBA-reactive substances/kg oil (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 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, N.Y., U.S.A.) at a speed setting of 7. The coarse emulsion was then sonicated with a Braun-Sonic U (B. Braun Biotech, Allentown, Pa., U.S.A.) at 4 °C for 3 min at maximum power and 0.5 duty cycle. The final emulsion droplet size was 0.91 mm as determined by laser light scattering (Mancuso and others 1999). The emulsion was diluted with whey, whey fractions, and/or buffer to a final lipid concentration of 10%. Thimerosal (1 mM) was added to prevent bacterial spoilage.

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, Calif., U.S.A.), a total of 3 times, after 3, 6, and 12 h of constant stirring at 5 °C. The protein content of the HMW fraction of whey (average 14.0 mg protein / mL) was measured using the biuret method (Chang, 1994). The low molecular weight (LMW) fraction of whey was isolated by ultra-filtration in a Molecular/Por Stirred Cell (PGC Scientific, Gaithersburg, Md., U.S.A.) using a DiaFlo YM3 (Amicon, Beverly, Mass., U.S.A.) 3,000 molecular weight cutoff membrane.

The HMW fraction of raw whey (25 mL in 50 mL Erlenmeyer flasks) was exposed to heat treatments of 60, 70, 80, and 90 °C for 15 min in a water bath with constant stirring. Heating time included time to reach the desired temperature. Protein unfolding after heat treatments were measured spectrophotometrically at 273 nm (Robyt and White, 1987) using a 20-fold dilution of the HMW fraction. No increase in absorbance was observed as a function of heating at wavelengths of 350 to 400 nm, indicating that the increase in absorbance at 273 nm was not due to protein aggregation and light scattering.

"Reactive" and "total" sulfhydryl content of the whey and continuous phase whey proteins were 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, without using a denaturant. To measure "total" sulfhydryls, 8 M urea was added to the whey (15 min, 25 °C) to completely unfold the whey proteins, measuring all sulfhydryls present. 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 (aqueous) phase whey or HMW fraction (2.0 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 was then centrifuged at 1000 × g for 5 min. Absorbance of the supernatant was determined at 412 nm. Turbidity was corrected for at 540 nm using the formula: $Abs_{412\ nm} - Abs_{540\ nm}$ (Taylor and Richardson 1980). Urea and butanol controls were performed and were not found to interfere with the assay. Concentrations of sulfhydryls were determined from a standard curve prepared using cysteine.

Residual phospholipids were isolated from HMW whey by solvent extraction with 3:2 hexane/isopropanol. HMW fraction whey (3 mL) and solvent (15 mL) were vortexed and the upper solvent phase was collected. After the extraction was performed a total of 3 times, the solvent was pooled, evaporated under nitrogen and resuspended into 3 mL phosphate buffer (50 mM, pH 7.0).

Lipid oxidation measurements

Emulsions (6 mL) were placed in test tubes (13 × 125 mm) and allowed to oxidize at 20 °C for up to 21 d. Controls contained the salmon oil emulsion and buffer only. Lipid peroxides (a modification of Shantha and Decker 1994) were measured by mixing emulsion (0.3 mL) with 1.5 mL of isooctane/2-propanol (3:1, v/v), by vortexing (10 seconds, 3 times), and isolation of the organic solvent phase by centrifugation at 1000 × g for 2 min. The organic solvent phase (200 ml) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 mL of 3.94 M ammonium thiocyanate and 15 mL of ferrous iron solution (prepared by mixing $BaCl_2$ and $FeSO_4$ to a final concentration of 0.132 and 0.144 M, respectively). The absorbance of the solutions were 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 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 followed by heating in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min then centrifuged (1000 × g) for 15 min. Absorbance was measured at 532 nm. Concentrations of TBARS were determined from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

Measurement of α-tocopherol

Levels of α-tocopherol were measured using an HPLC method (Hatam and Kayden 1979) consisting of a 3:1 acetonitrile:methanol mobile phase (1.5 mL/min.), an Alltech Econosil C18 10-micron column, Waters 470 scanning fluorescence detector (excitation wavelength of 298 nm and an emission wavelength of 340 nm), Waters 510 HPLC pump, and Millipore Millenium 2010 Chromatography Manager software. Alpha-Tocopherol was quantified using a standard curve made with α-tocopherol solutions in hexane.

Statistics

All experiments were performed on triplicate samples. Analysis of variance was performed and differences between means were determined using Duncan's multiple range test ($p < 0.05$; Snedecor and Cochran 1989).

Results and Discussion

Antioxidant activity of the various fractions of pasteurized whey

The antioxidant activity of whey and its low- and high-molecular-weight fractions in Tween 20™-stabilized salmon oil emulsions was determined by measurement of TBARS and lipid peroxides. All pasteurized whey fractions inhibited lipid oxidation (Figure 1) in comparison to controls that contained no whey. A sample containing only Thimerosal (added to minimize microbial growth) had no effect on oxidation rates (results not shown). Inhibition of oxidation was in the order of whole whey > HMW fraction whey > LMW fraction whey. After 10 d of oxidation, 10 % whole whey, HMW fraction, and LMW fraction whey inhibited oxidation 94.6, 86.3, and 58.0%, respectively. The HMW fraction represents the protein and phospholipid fraction of the whey, while the LMWF represents the lactose, mineral and other low-molecular-weight compounds. An isolated phospholipid fraction was found not to affect oxidation rates at a concentration equivalent to 10 % whey (by vol., data not shown). This inability of the isolated phospholipids to impact oxidation indicates that the whey proteins are primarily responsible for the antioxidant activity of the HMW fraction in a Tween 20™-stabilized salmon oil emulsion. The antioxidant activity seen in the LMW fraction may be due to unidentified antioxidants (Colbert and Decker 1991) or Maillard browning products (Browdy and Harris 1997). To focus on the antioxidative effect of whey proteins, the HMW fraction of whey was used for all further experiments.

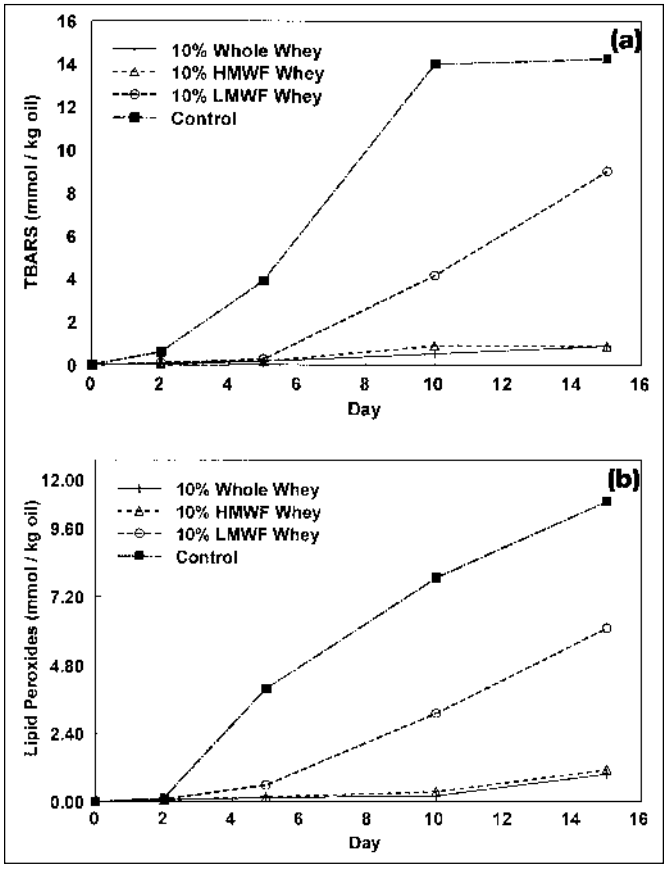

Figure 1—Influence of pasteurized whey and its high- (HMWF) and low- (LMWF) molecular-weight fractions (10% by vol) on the formation of thiobarbituric acid-reactive substances (a, TBARS) and lipid peroxides (b) in a 5% salmon oil emulsion stabilized by Tween 20

Effect of heat treatment on the properties of the HMW fraction of raw whey

Since whey protein from pasteurized skim milk has been heated, which is known to cause protein unfolding (Swaisgood 1996) and increase the antioxidative activity of whey (Taylor and Richardson 1980), the effect of heat treatment on the inhibition of lipid oxidation by the HMW fraction of raw whey was further evaluated. Heat treatments chosen were no heat, 60, 70, 80, and 90 °C for 15 min. This temperature profile represents a range in which whey proteins are known to unfold (Kinsella and Whitehead 1989). Figure 2 demonstrates that protein unfolding and exposure of aromatic amino acids (increase in UV absorbance at 273 nm) takes place during the heating of the HMW fraction from raw whey, with unfolding increasing with increasing temperature. These results confirm that the conformation of whey proteins in the HMW fraction was altered during heating (Morr and Ha, 1993).

Unfolding of whey proteins can be accompanied by an increase in exposure of reduced sulfhydryl groups, which were previously buried in the interior of the protein (Phillips and others 1994). Reduced sulfhydryls are known free radical scavengers (Darkwa and others 1998). The reactive and total reduced sulfhydryl content of the HMW fraction for each heat treatment are shown in Figure 3. With no heat, 31% of the total reduced sulfhydryls were reactive. With increasing heat treatment, this percent-

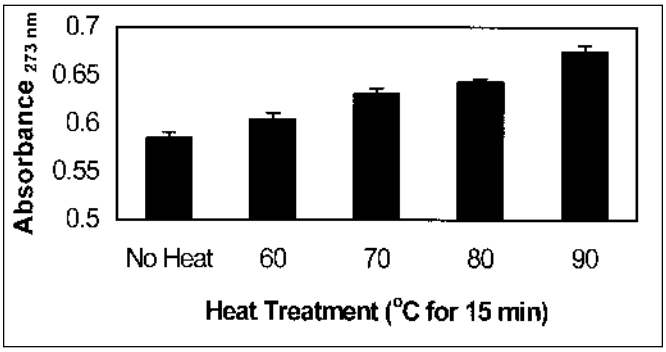

Figure 2—Influence of heat treatment on the unfolding of proteins in the HMW fraction of whey from raw milk

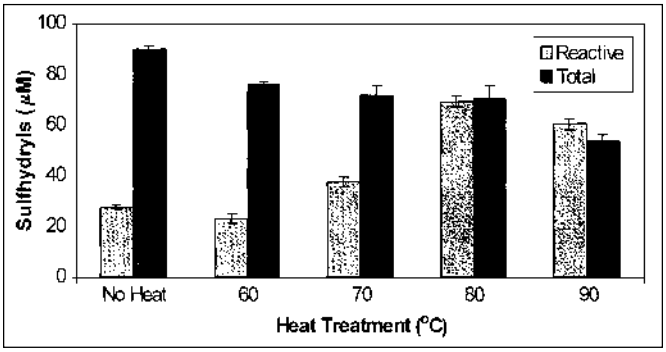

Figure 3—Influence of heat treatment on total and exposed reduced sulfhydryl concentrations on the HMW fraction of whey from raw milk

age significantly ($p < 0.05$) increased with the 80°C treatment, having essentially all its reduced sulfhydryls in the reactive form. As temperature was increased to 90 °C, a significant ($p < 0.05$) decline in total reduced sulfhydryl content of 40 % occurred, indicating a heat-induced oxidation of sulfhydryls which was also observed by Taylor and Richardson (1980). Thus, the optimum heat treatment for increasing reduced sulfhydryl exposure without decreasing total sulfhydryl was 80 °C.

Effect of heat treated HMW fraction of raw whey on oxidation of emulsions

Oxidation studies were carried out with the raw and heat-treated whey (10% by vol.) in Tween 20™-stabilized salmon oil emulsions. The formation of TBARS and lipid peroxides in the presence and absence of heated HMW fraction whey is shown in Figures 4a and 4b, respectively. The unheated and 60 °C heated HMW fractions were prooxidative. Whey protein isolate can also be prooxidative in menhaden oil emulsions (Donnelly and others 1998). The reasons for these observed prooxidative effects are unknown. Upon further heating, an inhibitory effect of the HMW fraction was observed with maximum antioxidant activity occurring in the 80 °C heated sample. The antioxidant effect of the 90 °C sample was 5 and 8 % less than the 80 °C heated sample at 5 d, as determined by TBARS and lipid peroxides, respectively.

Variations in the impact of the HMW fraction of whey on lipid oxidation in emulsions shows that the whey proteins can be prooxidative or antioxidative depending on their thermal history. With no heat or a mild heat treatment, the balance seems to lie with the prooxidative factors, while a stronger heat treatment shifts the balance and antioxidative factors become more important, thus outweighing any prooxidant activity.

Oxidation in salmon oil-in-water emulsions containing HMWF and α-tocopherol

If highly unsaturated oils are used in food, it is likely that more than one antioxidant will be required to adequately slow oxidation. Therefore, inhibition of lipid oxidation in the presence of the HMW fraction of whey and α-tocopherol was investigated to determine how these two antioxidants may interact. The salmon oil used in these experiments contained approximately 4 ppm α-tocopherol. An additional 40 ppm of α-tocopherol was added to the oil prior to emulsion formation. This relatively low α-tocopherol concentration was chosen so that some oxidation would occur during the course of the experiment, thus allowing the antioxidant effect of the HMW fraction to be observed when both antioxidants were used in combination. HMW fraction whey was added at 70 % of the assay volume to provide protein concentrations high enough to measure changes in sulfhydryl concentrations. The oxidation rate of salmon oil emulsions containing both 40 ppm α-tocopherol and the 80 °C heated HMW fraction (70 % by vol) is shown in Figure 5a (TBARS) and b (lipid peroxides). At 21 d, the HMW fraction and α-tocopherol combination, HMW fraction alone, and α-tocopherol alone inhibited TBARS 86, 59, and 19%, respectively. Alpha-Tocopherol initially (up to day 7) increased and then decreased lipid peroxide concentrations (Figure 5b). The ability of α-tocopherol to increase lipid peroxides is likely due to the antioxidant mechanism of α-tocopherol which

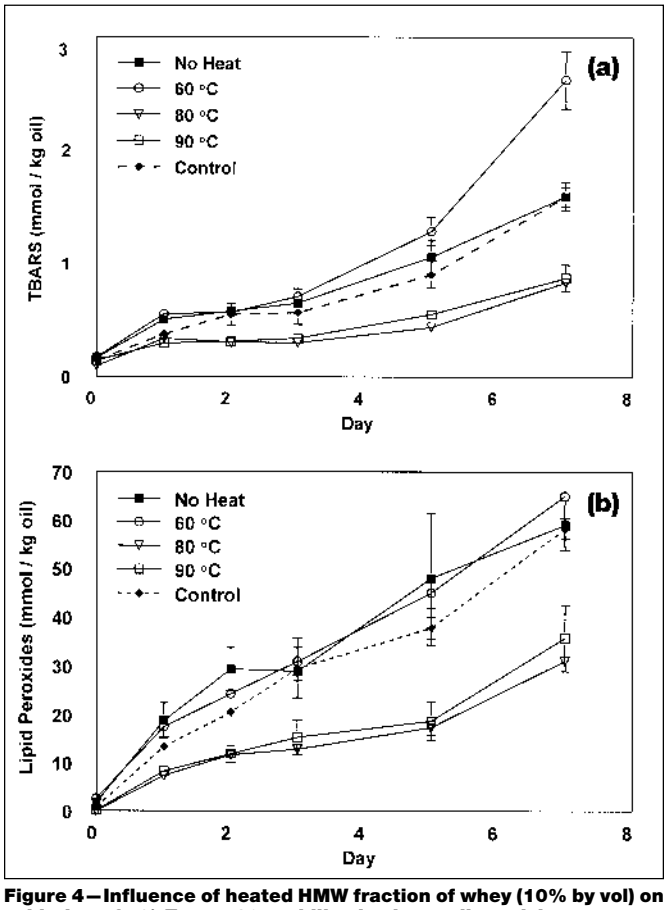

Figure 4—Influence of heated HMW fraction of whey (10% by vol) on oxidation of 5% Tween 20-stabilized salmon oil emulsions as measured by production of thiobarbituric acid-reactive substances (a, TBARS) and lipid perovides (b)

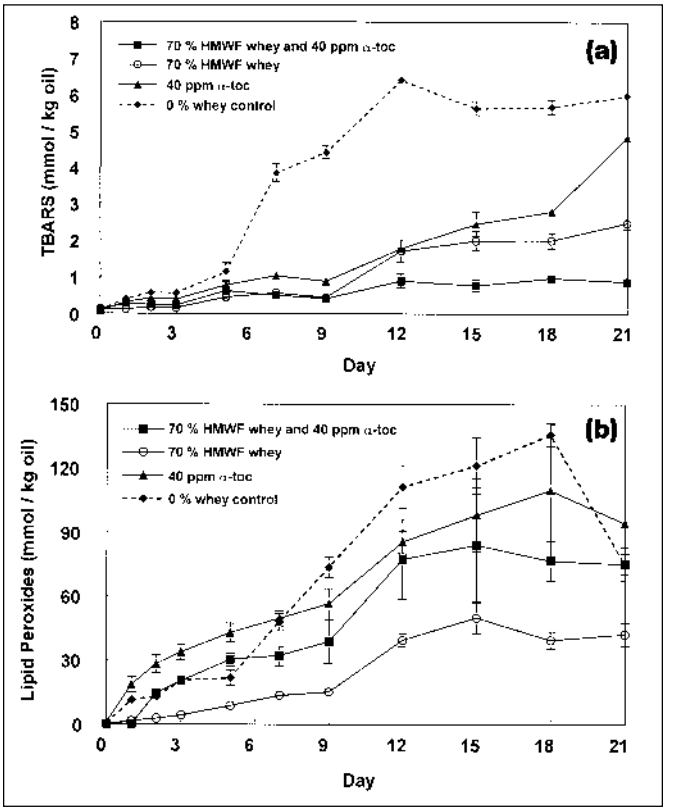

Figure 5—Effect of 80 °C heated HMW fraction whey (70% by vol), α-tocopherol (40 ppm) and their combination on the formation of thiobarbituric acid-reactive substances (a, TBARS) and lipid peroxides (b) in a 5% Tween 20-stabilized salmon oil emulsion. Control contained no added HMW fraction whey or α-tocopherol.

involves the donation of a hydrogen to a peroxyl radical resulting in lipid peroxide formation (Frankel 1998). While α-tocopherol increased peroxides, it inhibited formation of secondary lipid oxidation products as can be seen by its ability to inhibit TBARS formation (Figure 5a).

Figure 6 shows the α-tocopherol and continuous phase sulfhydryl concentrations in emulsion during oxidation. For the sample with 40 ppm α-tocopherol only, α-tocopherol concentrations declined faster (41 % reduction on day 7) compared to the α-tocopherol and HMW fraction combination (4 % reduction on day 7). Oxidation of sulfhydryls was faster than α-tocopherol with the sulfhydryl concentrations of both the HMW fraction only and HMW fraction and α-tocopherol combination being reduced by about 94 and 89 %, respectively, after 7 d. The fact that sulfhydryls are oxidized faster than α-tocopherol suggests that sulfhydryls are the primary antioxidant in the emulsion since they are preferentially oxidized over α-tocopherol. The ability of the HMW fraction to slow α-tocopherol oxidation in a salmon oil emulsion suggests that the HMW fraction may be able to regenerate oxidized α-tocopherol or that by reducing oxidation rates, and thus free radical production, it could slow α-tocopherol oxidation. Conversely, α-tocopherol did not slow sulfhydryl oxidation, suggesting that its ability to slow oxidation was not related to its ability to protect protein sulfhydryls. This could be due to its physical location (for example, α-tocopherol would scavenge lipid-soluble free radicals which may not oxidize continuous phase sulfhydryls) or because the lower reduction potential of α-tocopherol (−500 mV) would not be able to efficiently reduce oxidized sulfhydryls (reduction potential of oxidized cysteine = 920 mV; Buettner 1993).

Conclusions

THE HMW FRACTION OF PASTEURIZED WHEY, REPRESENTING primarily the proteins and phospholipids, has a greater antioxidative activity than the LMW fraction of pasteurized whey in salmon oil-in-water emulsions stabilized by a nonionic surfactant. Thermal processing of the HMW fraction of whey can result in both reactive sulfhydryl exposure and total sulfhydryl destruction. It is, therefore, important to utilize thermal processing conditions that maximize sulfhydryl exposure without causing total sulfhydryl destruction to obtain maximal antioxidant activity. When α-tocopherol and whey proteins were added in a salmon oil emulsion together, the protein sulfhydryl groups oxidized more quickly than α-tocopherol, suggesting that sulfhydryls are the primary antioxidants. These results show that whey proteins are effective antioxidants in salmon oil-in-water emulsions suggesting that they could provide an additional antioxidant defense system in food emulsions.

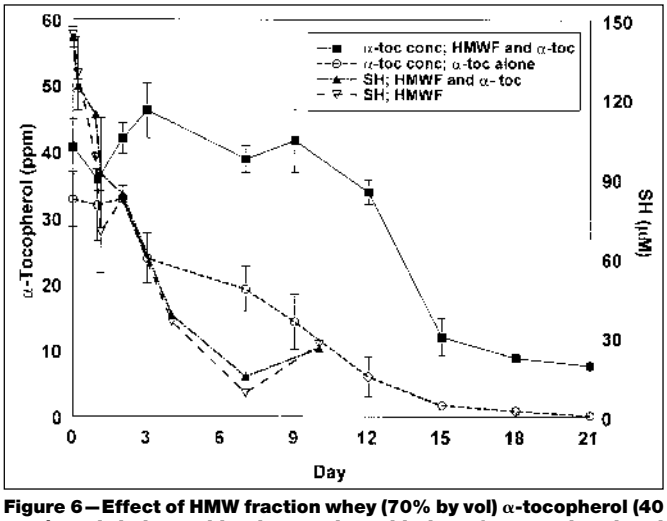

Figure 6—Effect of HMW fraction whey (70% by vol) α-tocopherol (40 ppm), and their combination on the oxidation of α-tocopherol and reduced sulfhydryls in a 5% Tween 20-stabilized salmon oil emulsion.

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