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Physical and Oxidative Stability of Fish Oil-in-Water Emulsions Stabilized with β-Lactoglobulin and Pectin

MARLY S. KATSUDA,[†,§] D. J. MCCLEMENTS,[#] LUCIA H. S. MIGLIORANZA,[†] AND ERIC A. DECKER*[,#]

Departmento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Londrina-PR, Brazil, and Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003

The oxidation of fatty acids can be inhibited by engineering the surface of oil-in-water emulsion droplets to decrease interactions between aqueous phase prooxidants and lipids. The objective of this research was to evaluate whether emulsions stabilized by a multilayer emulsifier systems consisting of β-lactoglobulin and citrus or sugar beet pectin could produce fish oil-in-water emulsions that had good physical and oxidative stability. Sugar beet pectin was compared to citrus pectin because the sugar beet pectin contains the known antioxidant, ferulic acid. A primary Menhaden oil-in-water emulsion was prepared with β-lactoglobulin upon which the pectins were electrostatically deposited at pH 3.5. Emulsions prepared with 1% oil, 0.05% β-lactoglobulin, and 0.06% pectins were physically stable for up to 16 days. As determined by monitoring lipid hydroperoxide and headspace propanal formation, emulsions prepared with the multilayer system of β-lactoglobulin and citrus pectin were more stable than emulsions stabilized with β-lactoglobulin alone. Emulsions prepared with the multilayer system of β-lactoglobulin and sugar beet pectin were less stable than emulsions stabilized with β-lactoglobulin alone despite the presence of ferulic acid in the sugar beet pectin. The lower oxidative stability of the emulsions with the sugar beet pectin could be due to its higher iron and copper concentrations which would produce oxidative stress that would overcome the antioxidant capacity of ferulic acid. These data suggest that the oxidative stability of oil-in-water emulsions containing omega-3 fatty acids could be improved by the use of multilayer emulsion systems containing pectins with low metal concentrations.

KEYWORDS: Omega-3 fatty acids; lipid oxidation; citrus pectin; sugar beet pectin; antioxidants; ferulic acid; emulsions; β-lactoglobulin

INTRODUCTION

Fish oils are rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are susceptible to oxidation because of the multiple double bonds in their carbon chain (*1*). The oxidative susceptibility of fish oil also depends on the presence of antioxidants and prooxidants found in the oil as well as the food matrix to which they are added. Transition metals such as iron or copper are naturally present in foods and may originate from raw materials, ingredients, processing equipment and packaging materials. Copper and iron are both common in foods; however, iron is usually found in higher amounts than copper. These transition metals promote lipid oxidation through their ability to generate free radicals from reactions such as lipid hydroperoxide decomposition (*2*). In order to maximize the oxidative stability of commercial oils, iron and copper concentrations in oil should be as low as possible with most specifications recommending <0.1 ppm and 0.02 ppm, respectively (*3*).

A number of studies have shown that oil-in-water emulsions containing lipid droplets encapsulated by multilayered coatings can be created using food grade surfactants and biopolymers (*4–8*). These multilayer coatings increase the physical stability of oil-in-water emulsions to stresses such as temperature, mechanical agitation, pH and the presence of multivalent ions (*4–8*). Multilayer coatings are typically produced through the electrostatic deposition of a charged biopolymer onto a lipid droplet covered with an emulsifier of opposite charge. These coatings can be produced by altering the pH so that it is below the protein pI (thus the protein is cationic) and higher than the hydrocolloid pK_a (thus the hydrocolloid is anionic (*9, 10*).

Whey proteins have been widely used as emulsifiers in the food industry. These proteins do not only produce physically stable oil-in-water emulsions (O/W), but they also inhibit lipid oxidation (*11*). When present on the surface of an oil-in-water

* Author to whom correspondence should be addressed [telephone (413) 545-1026; fax (413) 545-1262; e-mail edecker@foodsci.umass.edu].

[†] Universidade Estadual de Londrina.

[§] Present address: Universidade Tecnológica Federal do Parana, Food technology department, Medianeira, Paraná State, Brazil.

[#] University of Massachusetts.

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emulsion droplet, whey proteins can inhibit lipid oxidation at pH values below the isoelectric point (pI) of proteins through the production of a cationic surface which electrostatically repels cationic transition metals. Studies performed with salmon oil-in-water emulsions stabilized by various whey protein sources at pH 3 have produced emulsions with an oxidative stability in the following order: β-lactoglobulin $\geq$ α-lactalbumin $\geq$ whey protein isolates (*12–14*).

Pectins are anionic polysaccharides normally derived from citric fruits and apples that are used to increase viscosity and form food gels (*15*). Pectin is defined as a mixture of heteropolysaccharides predominantly composed of residues of methoxylated galacturonic acids (*15*). Pectins may be classified according to their degree of methoxylation as either high methoxyl pectin ($\geq$50%), which form gels under acid conditions or low methoxyl pectin ($<$50%), which form gels in the presence of calcium (*15, 16*). Sugar beet (*Beta vulgaris*) pectin is extracted from sugar beet pulp residue and is widely used as animal feed. Due to its poor gelation properties, the use of this pectin is restricted in the food industry. The poor gelation properties are mainly attributed to the presence of the acetyl ester group and to the reduced size of its molecules (*17, 18*). Sugar beet pectin also contains ferulic acid whose acid group is esterified to the oxygen on carbon 2 of arabinose or carbon 6 of galactose (*19*) thus leaving the hydroxyl group of ferulic acid free to potentially interact with free radicals.

Lipid oxidation in oil-in-water emulsions is highly dependent on the interfacial characteristics of the lipid droplets since this is the location where transition metals and lipid hydroperoxides interact to form free radicals that can attack other unsaturated fatty acids to continue the oxidative reactions (*20, 21*). Multilayer coatings have the potential to decrease lipid oxidation rates due to their ability to alter both the emulsion droplet charge and thickness of the interfacial region, both factors that can alter metal-hydroperoxide interactions (*6*). For example, a multilayer emulsion stabilized by a Lecithin/chitosan coating had greater oxidative stability than one stabilized by a Lecithin coating alone during 3 weeks of storage at room temperature (*6*).

The objective of this work was to determine if the physical and oxidative stability of Menhaden oil-in-water emulsions could be increased by coating lipid droplets with β-lactoglobulin and pectin (from either sugar beet or citrus). These two different types of pectin were tested since sugar beet pectin contains ferulic acid which has antioxidative properties, whereas citrus pectin does not. If the sugar beet pectin can increase the oxidative stability of oil-in-water emulsions susceptible to lipid oxidation, this could provide a protection system that could increase the utilization of lipids such as omega-3 fatty acid oils. Emulsions prepared with Tween 20, a nonionic emulsifier used in foods was used to evaluate the role of continuous phase citrus and sugar beet pectin on lipid oxidation reactions.

MATERIALS AND METHODS

Materials. Monobasic and dibasic sodium phosphate, ferrous sulfate, cumene hydroperoxide, propionaldehyde, sodium azide, Tween 20, and citrus pectin (esterification level of 59%) were from Sigma-Aldrich Co. (St. Louis, MO). Sugar beet pectin (esterification level of 50%) was obtained from Herbstreith & Fox (Neuenbürg,Germany). β-Lactoglobulin was obtained from Davisco Foods International (BIOPURE β-lactoglobulin lot JE 003-6-922, Le Sueur, MN) and was composed of 93.8% of β-betalactoglobulin, 5.2% water, 1% of fat and 1.8% ash. Deodorized, refined and bleached Menhaden oil without added antioxidants was supplied by Omega Protein (Reedville, VA) and contained 10−17% of EPA and 7−12% of DHA. Oil was stored in the dark at −80 °C and thawed in cold tap water immediately before use. Distilled and deionized water was used in all studies. All other reagents were of analytical grade or better.

Methods. *Preparation of the Stock Emulsion.* Emulsifier solutions contained 0.5% (w/w) of β-lactoglobulin or 1% (w/w) of Tween 20 in 5 mM sodium phosphate buffer at pH 7. The β-lactoglobulin solution was mixed into the buffer for 4 h and then stored in the refrigerator (6 $\pm$ 2 °C) for 12 h in order to ensure hydration. Before the emulsion was prepared, the solution was brought to room temperature. The Tween 20 solution was prepared the same day as emulsion preparation. Stock emulsions were prepared by prehomogenizing Menhaden oil (10%) with the emulsifier solutions (90%) with the use of a high-speed mixer (Biospec Product, Inc., Switzerland) for 2 min. These prehomogenized emulsions were then further homogenized in a Microfluidizer (model M-110L Microfluidizer Processor, Microfluidics, Newton, MA) for 3 passes at a pressure of 68 MPa. The emulsions were kept cold between passes through the homogenizer in a cold-water bath to minimize oxidation. After homogenization, 0.01% (w/w) of sodium azide was added as a microbial preservative. To increase the physical stability of the primary emulsion, the β-lactoglobulin-stabilized Menhaden oil-in-water emulsions were heated at 80 °C for 15 min to promote sulfhydryl cross-links between β-lactoglobulin at the emulsion droplet surface (*22*). Immediately after heating, the emulsions were cooled in cold tap water.

Primary emulsions were prepared by diluting the stock emulsion in 5 mM sodium phosphate buffer solution at pH 7 at the proportion of 1:10 (w/w) while slowly shaking for 45 min. Secondary emulsions were prepared by the method of Suhr et al. (*23*) with modifications. In short, secondary emulsions were prepared by slowly diluting the stock emulsion prepared with β-lactoglobulin into 5 mM phosphate buffer solution containing either citrus (CP Blg) or sugar beet (BP Blg) pectin at concentrations ranging from 0.01 to 0.5% (w/w). The Tween 20 emulsion was also mixed with the citrus (CP Tw) or sugar beet (BP Tw) pectin using the same procedure. Following addition of pectin to the emulsions, the pH was adjusted to 3.5 using 1 and 0.1 N HCl. The emulsions were stored in glass test tubes in the dark without stirring at 37 °C for physical and chemical analyses.

Evaluation of the Physical Stability of the Emulsion. To measure ζ potential, emulsions were diluted into 5 mM phosphate buffer (pH 3.5) at a ratio of 1:100 (v/v) and then injected into a Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, U.K.). The results were obtained from the average of 10 readings. Emulsion droplet size was obtained using a Mastersizer static laser light dispersion instrument using a refractive index of 1.43 (Malvern MSS, Malvern Instruments, Worcestershire, U.K.). In order to avoid the multiple scanning effect, emulsions were diluted at a ratio of 1:200 (v/v) using 5 mM sodium phosphate buffer (pH 3.5). Both emulsion droplet diameter (d_{32}) and volume (d_{43}) were determined.

Measurement of the Lipid Oxidation. Lipid hydroperoxides were determined by mixing emulsion samples (0.3 mL) with 1.5 mL of isooctane/2-propanol (3:1 v/v) and vortexing for 10 s a total of three times. After centrifugation for 2 min at 2000*g* (Centrific centrifuge, Fisher Scientific), 0.20 mL of the clear upper layer was collected and hydroperoxides were quantitated using a modified method of Shanta and Decker (*24*). In short, the sample extract (0.2 mL) was mixed with 2.8 mL of methanol/1-butanol (2:1 v/v), 15 μL of 3.94 M ammonium thiocyanate solution and 15 μL of 0.072 M ferrous ion solution (prepared through the mixture of $BaCl_2$ 0.132 M and $FeSO_4$ 0.144 M). After 20 min of incubation at room temperature, absorbance was measured at 510 nm. Hydroperoxides concentrations were determined using a cumene hydroperoxide standard curve.

Propanal (a breakdown product of omega-3 fatty acids) analysis was performed on 1 mL of emulsion that was placed into 10 mL headspace vials sealed with PTFE/silicone septa. Headspace propanal concentration was measured using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). A 30 m $\times$ 0.32 mm Equity DB-1 column (Supelco, Bellefonte, PA) with a 1 μm film thickness was used for separations. Each sample was shaken and heated at 55 °C in the autosampler heating block for 13 min. A 50/30 μm DVB/Carboxen/PDMS solid phase microextraction (SPME) fiber needle (Supelco, Bellefonte, PA) was injected into the headspace of the sample vial for 1 min to adsorb volatiles and then injected into the

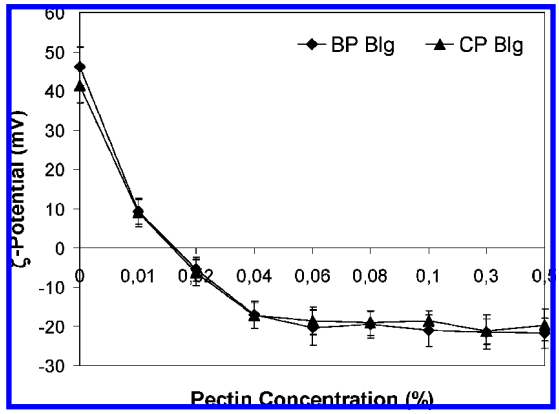

Figure 1. Effect of different citrus (CP) (▲) and sugar (SB) beet (◆) pectin concentrations on the zeta potential of Menhaden oil-in-water emulsions stabilized by β-lactoglobulin at pH 3.5. Data represents mean ($n = 3$) ± standard deviation. Some error bars lay within data points.

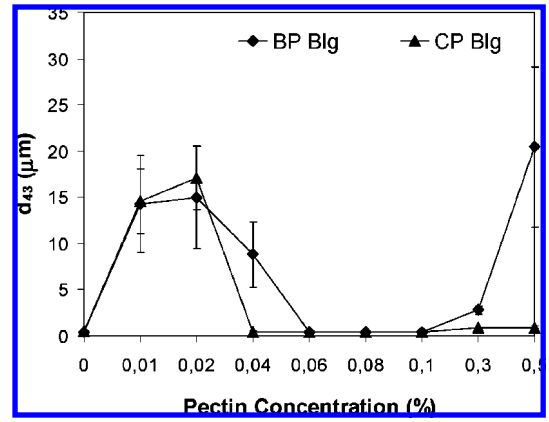

Figure 2. Influence of the different citrus (CP) (▲) and sugar (SB) beet (◆) pectin concentrations on the average particles diameter (d_{43}) of Menhaden oil-in-water emulsions stabilized with β-lactoglobulin at pH 3.5. Data represents mean ($n = 3$) ± standard deviation. Some error bars lay within data points.

injector port at 250 °C for 3 min. The gas chromatograph ran for 10 min at 70 °C for each sample with a total helium flow rate of 1.5 mL/minute and a detector temperature of 250 °C. Propanal concentrations were determined from peak areas using a propanal standard curve prepared from propanal in phosphate buffer.

Determination of Metals Concentrations in Pectin Samples. Iron and cooper concentrations in citrus and sugar beet pectins were determined by atomic absorption spectrophotometry. The analytical procedures were performed according to the AOAC (*25*). The results were determined from the average of 3 readings for each sample.

Statistical Analysis. All analyses were performed on triplicate samples. Differences between samples were determined using the Student's test at a 5% of significance level (*26*).

RESULTS AND DISCUSSION

Initially, we carried out a series of preliminary experiments to identify the optimum citrus and sugar beet pectin concentrations required to produce physically stable emulsions containing lipid droplets encapsulated within β-lactoglobulin/pectin coatings. Different amounts of the pectins (0.01 to 0.5% w/w) were added to β-lactoglobulin-stabilized oil-in-water emulsions at pH 7.0. The system was then adjusted to pH 3.5 so that the adsorbed proteins were below their isoelectric point, which promoted electrostatic deposition of the anionic pectin molecules onto the cationic β-lactoglobulin-coated lipid droplets (*9, 27*). The ζ potential of the primary emulsion in the absence of pectin ranged from +42 to +45 mV (**Figure 1**) which is expected for emulsions stabilized with β-lactoglobulin at pH 3 (*4*). Upon the addition of the pectin, the ζ potential of the emulsion droplets changed from positive to negative showing that the anionic pectins deposited onto the layer of β-lactoglobulin molecules on the lipid droplet surfaces. For both sugar beet and citrus pectin, ζ potential values became relatively constant at pectin concentrations above 0.04%. These results indicate that the concentration of 0.06% was sufficient to saturate the surface of the emulsion droplets. The ζ potential results demonstrated in this study were similar to those reported for corn oil emulsions stabilized by β-lactoglobulin and citrus pectin at pH 3 (*4*).

Beet pectin concentrations below 0.06% (w/w) and citrus pectin concentrations below 0.04% promoted an increase in mean particle size, i.e., the d_{43} values were higher than those in the primary emulsion containing no added pectin ($d_{43} = 0.45$ μm) (**Figure 2**). This increase in mean particle size can be attributed to the ability of the pectin molecules to cause charge neutralization and bridging flocculation (*27*). Secondary emulsions prepared with beet pectin concentrations between 0.06% and 0.1% and citrus pectin concentrations between 0.04% and 0.1% had a mean particle size similar to the primary emulsion ($d_{43} = 0.41$ μm for both pectins). At beet pectin concentrations above 0.1%, mean particle size increased with d_{43} being greater than 2.79 μm. Increasing citrus pectin concentrations above 0.1% caused a smaller increase in particle size compared to samples with beet pectin. The increase in particle at high concentrations of pectin is likely due to depletion flocculation. The difference between the two pectins may be due to differences in the molecular weights, since it is known that the critical flocculation concentration decreases with increasing molecular weight for nonadsorbed biopolymers that induce depletion flocculation (*28*). Since 0.04% of both sugar beet and citrus pectin produced small emulsion droplets that were saturated with pectin at the droplet interface, this concentration was chosen for emulsion stability studies.

β-lactoglobulin stabilized emulsions with and without citrus or sugar beet pectin coatings showed no change in mean particle diameter (d_{32}) after 16 days of storage at 37 °C at pH 3.5 (**Figure 3**). The results for the β-lactoglobulin only treatment were similar to those found by Hu et al. (*28*), where the mean particle diameter remained between 0.29 to 0.35 μm (d_{32}) for corn oil emulsions stabilized by whey protein isolates at pH 3 during 4 days of storage. Emulsions stabilized by β-lactoglobulin alone showed a significant increase in d_{43} values after 16 days of storage. Emulsions stabilized by β-lactoglobulin and pectins were similar to previous studies (*4*) with d_{43} ranging between 0.35 and 0.38 μm and not changing during the 16 days of storage. Emulsions stabilized by Tween without citrus or sugar beet pectin at pH 3.5 had similar d_{32} values at 0 and 16 days of storage. However, emulsions stabilized by Tween 20 had a decrease in d_{32} values at 0 and 16 days of storage in the presence of citrus or sugar beet pectin. No differences in relation to d_{43} values were observed in the emulsions stabilized by Tween 20. The d_{32} parameter is frequently associated with the average surface area of particles present in the continuous phase per volume unit in the emulsion. The d_{43} parameter is more sensitive to the presence of large-size particles than d_{32}, thus allowing detection the flocculation. There is no doubt that Tween is an outstanding stabilizer, allowing the preparation of emulsions with no flocculation problems or creaming, being therefore adopted as control in oxidation studies (*22*). It is not clear why the d_{32} values of the Tween-stabilized emulsions decreased during storage in the presence of the citrus or sugar beet pectins. However, it should be noted that the decrease in d_{32} values in

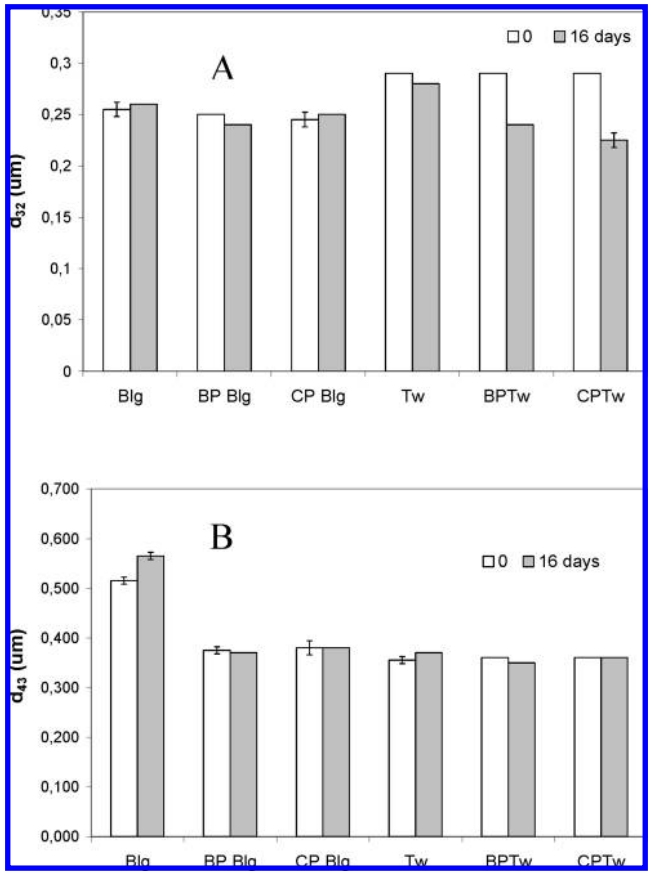

Figure 3. Average particle diameter (**A**, d_{32}; **B**, d_{43}) of emulsions at times 0 and 16 days at pH 3.5. Emulsions were stabilized by β-lactoglobulin alone (Blg), β-lactoglobulin plus sugar beet pectin (BP Blg), β-lactoglobulin plus citrus (CP Blg), Tween 20 alone (Tw), Tween 20 plus sugar beet pectin (BP Tw) and Tween 20 plus citrus pectin (CP Tw). Data represents mean ($n = 3$) $\pm$ standard deviation. Some error bars lie within data bar.

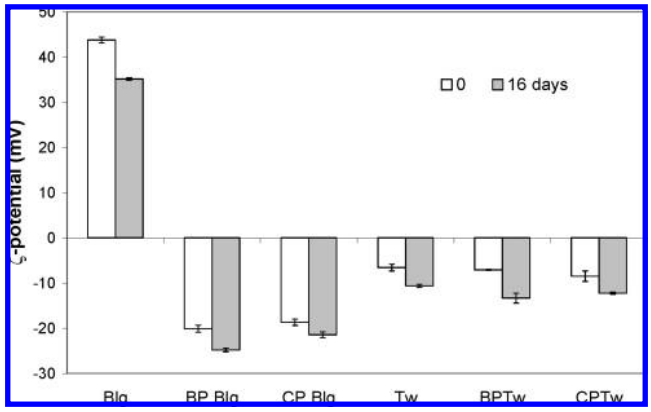

Figure 4. Zeta potential of emulsions at times 0 and 16 days at pH 3.5. Emulsions were stabilized by β-lactoglobulin alone (Blg), β-lactoglobulin plus sugar beet pectin (BP Blg), β-lactoglobulin plus citrus (CP Blg), Tween 20 alone (Tw), Tween 20 plus sugar beet pectin (BP Tw) and Tween 20 plus citrus pectin (CP Tw). Data represents mean ($n = 3$) $\pm$ standard deviation. Some error bars lie within data bar.

these samples occurred shortly after the lipids began to oxidize (d_{32} values during this period are not shown).

The stability of the emulsion droplets was also monitored through changes in their electrical charge during 16 days of storage (**Figure 4**). The ζ potential of all of the emulsions became slightly more negative during storage ($p \leq 0.05$). This

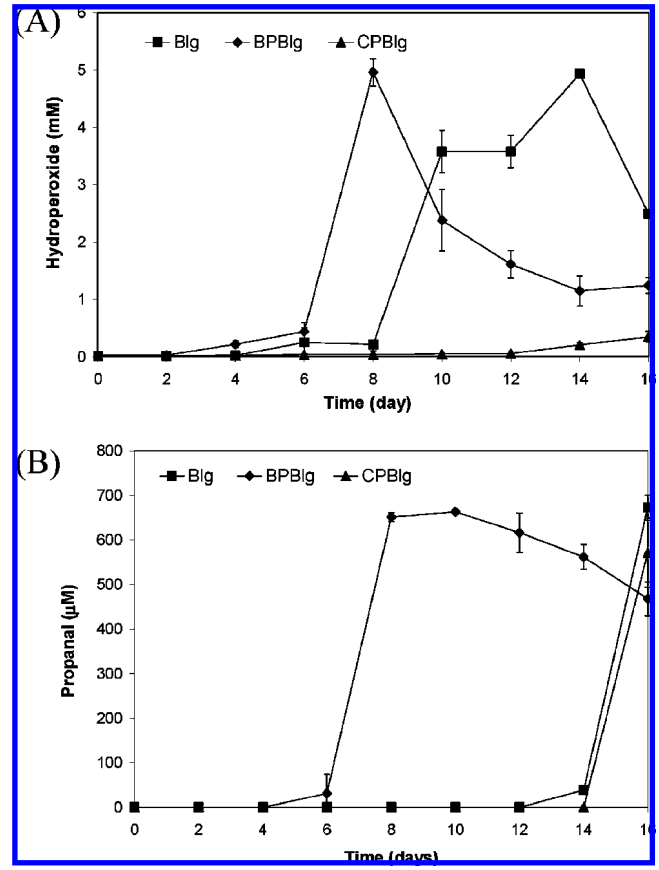

Figure 5. Formation of lipid hydroperoxides (**A**) and headspace propanal (**B**) in Menhaden oil-in-water emulsions (pH 3.5 and 37 °C) stabilized with β-lactoglobulin alone (Blg, ■), β-lactoglobulin plus sugar beet pectin (BP Blg, ◆), β-lactoglobulin plus citrus (CP Blg, ▲). Data represents mean ($n = 3$) $\pm$ standard deviation. Some error bars lay within data points.

change in ζ potential could be due to formation of anionic species such as free fatty acids which are generated from the hydrolysis of triacylglycerols at low pH values (*30*) or could be due to the loss of cationic species such as amines that will react with aldehydes arising from lipid oxidation to cause a loss of charge (*31*).

The oxidative stability of oil-in-water emulsions can be evaluated by measuring the amount of storage time before the formation of lipid oxidation markers increases rapidly. This lag phase for this work was defined as the amount of time required for the oxidation markers to be statistically greater than time 0 values. **Figure 5** shows the formation of lipid hydroperoxides and headspace propanal in Menhaden oil-in-water emulsions stabilized with β-lactoglobulin with and without citrus and beet pectin multilayers. In the emulsions stabilized with β-lactoglobulin alone, lipid hydroperoxides increased after 4 days of storage while headspace propanal increased after 12 days of storage. The longer lag phase for propanal is not unexpected since lipid hydroperoxides must decompose before propanal is formed by β-scission reactions. Secondary emulsions made with citrus pectin had longer phases for both hydroperoxide (12 days) and propanal (14 days) formation compared to the primary emulsion. The citrus pectin secondary emulsion was more oxidatively stable than the β-lactoglobulin-stabilized emulsion even though the surface charge of the secondary emulsion was anionic and thus would attract prooxidative metals. This suggests that the thicker emulsion droplet interfacial membrane was able to inhibit metal-lipid interactions and thus decrease lipid

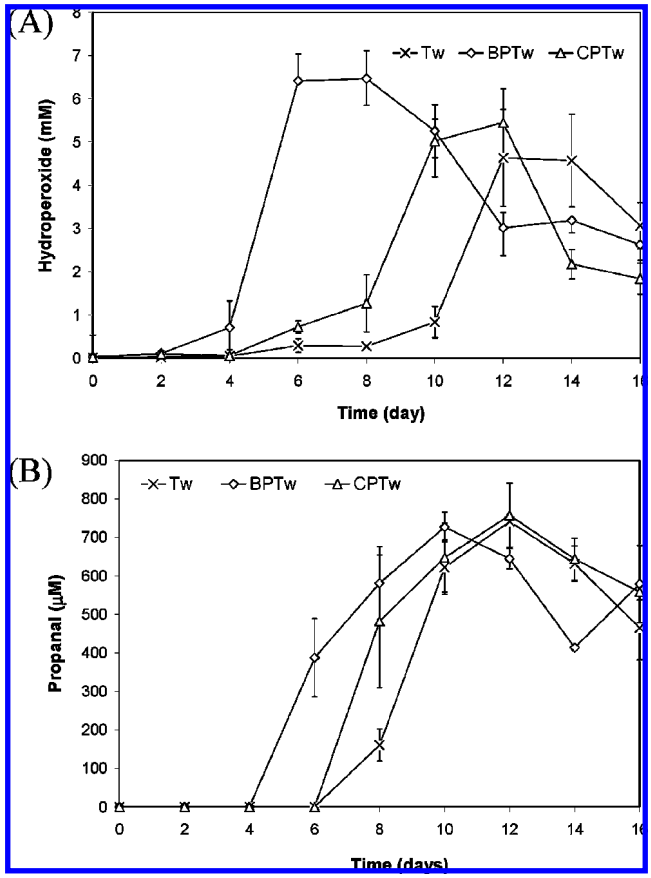

Figure 6. Formation of lipid hydroperoxides (**A**) and headspace propanal (**B**) in Menhaden oil-in-water emulsions (pH 3.5 and 37 °C) stabilized with Tween 20 alone (Tw, ×), Tween 20 plus sugar beet pectin (BP Tw, ◇), and Tween 20 plus citrus (CP Tw, △). Data represents mean ($n = 3$) ± standard deviation. Some error bars lay within data points.

oxidation rates. Greater oxidative stability of an anionic multilayer emulsion has also been reported by Ogawa et al. (*6*) who studied a Lecithin-chitosan-pectin tertiary emulsion which had pectin as the outermost layer. It was expected that the secondary emulsions prepared with beet pectin would be more oxidatively stable since beet pectin contains the antioxidant ferulic acid (*32, 33*). However, the secondary emulsions stabilized with beet pectin were less oxidatively stable than either the primary emulsion or the secondary emulsion prepared with citrus pectin. The lag phases for lipid hydroperoxide and headspace propanal formation in the beet pectin secondary emulsions were 2 and 4 days, respectively.

To evaluate the impact of pectins not associated with emulsion droplets on the oxidative stability of the Menhaden oil-in-water emulsions, pectins were added to Tween 20 stabilized emulsions. This system was chosen because Tween 20 produces weakly anionic emulsion droplets and thus the pectins do not absorb to the emulsion droplet surface. The lag phase for the formation of lipid hydroperoxide and headspace hexanal was similar for both the Tween 20 (4 days and 6 days, respectively) and the Tween 20-citrus pectin (4 days and 6 days, respectively) emulsions (**Figure 6**). However, the rate of both lipid hydroperoxide and propanal formation after the lag phase was greater for the Tween 20-citrus pectin than the Tween 20 alone. As was observed in the β-lactoglobulin-stabilized emulsions, the beet pectin accelerated lipid oxidation with the lag phases of lipid hydroperoxide and headspace propanal formation being only 2 and 4 days, respectively. Continuous phase pectin has been reported to inhibit lipid oxidation in oil-in-water emulsions when oxidative reactions were measured by oxygen consumption (*34*). The antioxidant activity of pectin has been postulated to be due to its ability to chelate metals and possibly scavenge free radicals (*34*).

It is unclear why the sugar beet pectin was prooxidative in the Menhaden oil-in-water emulsions especially since it contains ferulic acid a known antioxidant (*30*). The inability of the beet pectin to inhibit oxidation is even more surprising since the partitioning of antioxidants at the surface of an emulsion droplet is thought to be an effective technique to inhibit lipid oxidation (*29*). One possible reason for the prooxidant activity of the beet pectin was that it contains promoters of lipid oxidation such as transition metals. To determine if this was a possibility, the concentrations of iron and copper in both sugar beet and citrus pectin was determined. The commercial sugar beet pectin was found to contain over 14-fold more iron (1.91 ± 0.02 ppm vs 0.13 ± 0.00 ppm iron) and 2-fold more copper (0.08 ± 0.00 ppm vs 0.04 ± 0.00 ppm copper) than the citrus pectin. Since transition metals are strong prooxidants in oil-in-water emulsions (*29*), the presence of high concentrations of iron and of copper in sugar beet pectin would likely explain why lipid oxidation rates were highest in both the β-lactoglobulin and Tween 20 emulsions containing sugar beet pectin. These high transition metals concentrations would rapidly promote lipid oxidation and possibly overcome the antioxidant protection provided by ferulic acid.

Physically stable Menhaden oil-in-water emulsions were prepared using electrostatic deposition of pectin onto β-lactoglobulin. When the bilayer was made from β-lactoglobulin and citrus pectin the oxidative stability of the Menhaden oil was improved compared to the β-lactoglobulin alone. However, sugar beet pectin caused an acceleration of lipid oxidation when used in a bilayer system with β-lactoglobulin, presumably due to its high transition metal concentrations. This research suggests that pectins with low metal concentrations could be used to inhibit the oxidation of omega-3 fatty acids in fish oils.

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