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Role of Continuous Phase Anionic Polysaccharides on the Oxidative Stability of Menhaden Oil-in-Water Emulsions

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The antioxidant role of selected polysaccharides was studied in the continuous phase of a Menhaden oil-in-water emulsion coated by polyoxyethylene(23) lauryl ether (Brij 35) at neutral pH. The addition of low-methoxyl (LM) and high-methoxyl (HM) pectin (0.02−0.1 wt %) reduced the formation of lipid hydroperoxides and thiobarbituric acid reactive substances with an inhibition that increased with increasing polysaccharide concentration in the continuous phase. α-Carrageenan and sodium alginate were less effective antioxidants than pectin and were prooxidative under certain conditions. None of the polysaccharides impacted the physical properties of the emulsions as determined by droplet particle size ($d_{43} \sim 0.32$ μm) and creaming index. LM and HM pectins had higher iron-binding capacities as compared to α-carrageenan and sodium alginate, which may relate to their higher antioxidant activities. These results suggest that the addition of anionic polysaccharides to the continuous phase of oil-in-water emulsions could be used to increase the oxidative stability of oil-in-water emulsions and thus prolong shelf life.

KEYWORDS: Lipid oxidation; emulsion; polysaccharide; antioxidants; Menhaden oil; ω-3 fatty acids

INTRODUCTION

The benefits of consuming long chain polyunsaturated fatty acids (PUFAs), for example, the inhibition of cardiovascular disease, cancer, and inflammatory disease, have been widely reported (*1*−*3*). However, PUFAs are vulnerable to oxidation during storage, which creates primary and secondary oxidation products that negatively impact the flavor, color, nutrient value, and functionality of foods and food components. In addition, lipid oxidation products are cytotoxic and genotoxic and thus detrimental to the health of consumers (*4*). Examples of cytotoxic lipid oxidation products that are of health concern include lipid peroxides, unsaturated aldehydes and malonaldehydes, and several cholesterol oxidation products (*5*, *6*).

Several studies have demonstrated that oil-in-water food emulsion systems in which the lipid portion is dispersed as miniscule droplets within an aqueous continuous phase can be effective vehicles for delivering bioactive lipids such as PUFAs into foods. The susceptibility of these emulsified lipids to oxidation depends on the degree of unsaturation of the fatty acids, the physical properties of the emulsion droplets in relation to the surrounding molecular environment, and the interactions with other molecules (e.g., prooxidants and antioxidants) in the food system. For example, if the molecular environment (e.g., pH above the pI of protein-stabilized oil-in-water emulsions) or composition (e.g., presence of free fatty acids) produces a negative charge on the emulsion droplet surface, the oxidative stability of the emulsion is low since positively charged transition metals are attracted to the emulsion droplet surface where they can readily interact with lipids (*7*). One strategy to inhibit lipid oxidation in oil-in-water emulsions is to use food additives that can bind transition metals and partition them away from the emulsion droplet surface. For example, proteins dispersed in the continuous phase of oil-in-water emulsions can inhibit deleterious oxidation reactions by a combination of metal chelation and free radical scavenging (*8*, *9*).

Polysaccharides have been extensively used as thickening, stabilizing, and gelling agents in the food industry. As stabilizers, polysaccharides can enhance the physical stability of emulsion systems by increasing the viscosity of the aqueous phase and inhibiting coalescence. The antioxidant ability of polysaccharide in emulsion systems has also been reported (*10*, *11*). Xanthan, which is used in many salad dressing oil-in-water emulsion applications, has been reported to exhibit an antioxidant activity in soybean oil-in-water emulsion encapsulated by cyclodextrin through unknown mechanisms. One hypothesis of the antioxidant mechanism of xanthan is that its pyruvate side chain residues are able to chelate metal ions and inhibit lipid oxidation in oil-in-water emulsions (*12*). Gum Arabic, which has covalently bound peptide moieties attached on its main backbone, has also been shown to have an antioxidant activity in a methyl linoleate-in-water emulsion stabilized by casein (*13*).

It is not surprising that anionic polysaccharides can act as chelators since the acid groups can bind metals. Even nonionic polysaccharides have been reported to form complexes with transition metal ions by the displacement of H_2O molecules in the solvation sphere of the cations by −OH groups of the polyols. The formation of metal−polysaccharide complexes increases as the polysaccharide concentrations increase (*14*, *15*). However, a high concentration of polysaccharides in the continuous phase of

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an emulsion system can result in physical instability due to the depletion flocculation. Anionic polysaccharides can also negatively impact the physical stability of an oil-in-water emulsion system because they are often associated with metals that can promote oxidation (*16*).

The purpose of this research is to better understand the concentrations of anionic polysaccharides in oil-in-water emulsions that can inhibit lipid oxidation without negatively impacting the physical stability. Anionic polysaccharides to be tested include low-methoxyl (LM) pectin, high-methoxyl (HM) pectin, carrageenan, and sodium alginate (Alg). To avoid confusion because of the prooxidant activity of metals associated with the anionic polysaccharides, treatments were performed to remove endogenous metals prior to testing the antioxidant activity. The results of this study will provide a better understanding of the antioxidative or prooxidative activity of selected anionic polysaccharides in oil-in-water emulsion systems, which could expand their utilization in the food industry to control oxidative deterioration.

MATERIALS AND METHODS

Materials. Deodorized, refined, and bleached Menhaden oil without added antioxidants was supplied by Omega Protein (Reedville, VA) and contained 10−17% EPA and 7−12% DHA. Oil was stored in the dark at −80 °C and thawed in cold tap water immediately before use. Polyoxyethylene(23) lauryl ether (Brij 35) was acquired from Aldrich Chemical Co., Inc. (Milwaukee, WI). LM pectin (esterification level of 37%) and HM pectin (esterification level of 70%) were obtained from Herbstreith & Fox (Neuenburg, Germany). α-Carrageenan was kindly donated by FMC BioPolymer (Philadelphia, PA). Alg, 2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), imidazole, fluorescein sodium salt, ethylenediaminetetraacetic acid (EDTA), 2-thiobarbituric acid (TBA), sodium acetate, ferrous chloride, and Chelex-100 were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other reagents were of high-performance liquid chromatography grade or purer.

Solution Preparation. In a previous study, Nuchi and co-workers (*17*) found that pretreatment of buffer solutions with Chelex resin to remove transition metals decreased oxidation rates in Tween 20-stabilized salmon oil-in-water emulsions. Therefore, metals were removed from all buffers used in this study by gentle mixing of 1 g of Chelex-100 within 500 mL of buffer for 24 h. The buffer was then separated from precipitated Chelex-100 by decantation.

Stock surfactant solution for emulsion preparation was prepared by dispersing surfactant (0.5 wt % Brij 35) in phosphate buffer (10 mM) adjusted to pH 7.0 with 1 M HCl. Stock polysaccharide solutions (HM pectin, LM pectin, Alg, or α-carrageenan) were prepared by dispersing 2 wt % of polysaccharide in 10 mM phosphate buffer (pH 7.0) and stirring overnight to ensure complete dispersion. Previous studies have shown that anionic polysaccharides can contain high levels of contaminating metals (*16*). Therefore, endogenous metals were also removed from polysaccharide stock solutions by adding 2 g Chelex-100/L polysaccharide stock solution, stirring overnight, and then separating the polysaccharide solution from the Chelex by decanting.

Preparation of Emulsion. An oil-in-water emulsion was prepared by homogenizing 5 wt % Menhaden oil and 95 wt % stock Brij 35 surfactant solution using a high-speed blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min followed by further particle size reduction with a Microfluidizer (model M-110 L Microfluidizer Processor, Microfluidics, Newton, MA) for three passes at a pressure of 68 MPa. The emulsions were kept in an ice container over the whole procedure to minimize oxidation. After homogenization, 0.04 wt % NaN_3 was added as an antibacterial agent, and the emulsion was adjusted to pH 7 by adding 1 M HCl. This emulsion was referred to as the stock emulsion.

A portion of the stock emulsions was diluted with polysaccharide solutions and/or buffer to achieve the final desired concentrations of 2.5 wt % Menhaden oil, 0.25 wt % Brij 35, and polysaccharide concentrations of 0, 0.02, 0.05, and 0.1 wt %. The final emulsions were adjusted to pH 7.0 using 0.1 M HCl and were stored in the dark at 15 °C. The ζ-potential (particle electrophoresis), particle size (static light scattering), and creaming stability (visual observation) of the final emulsions were determined using experimental methods described in the previous papers (*18*, *19*).

Table 1. TEs (μM) of LM Pectin, HM Pectin, Carrageenan, and Alg at Different Concentrations as Determined by the ORAC

	polysaccharide concentration		
	0.02 wt %	0.05 wt %	0.1 wt %
LM pectin	2.05 ± 0.04 a	2.00 ± 0.06 a	2.07 ± 0.04 a
HM pectin	1.95 ± 0.07 bc	1.98 ± 0.06 a	2.06 ± 0.06 a
carrageenan	1.82 ± 0.01 c	1.79 ± 0.01 c	1.74 ± 0.10 c
Alg	1.79 ± 0.01 c	1.80 ± 0.01 c	1.80 ± 0.02 c

Measurement of Oxidation Parameters. Lipid hydroperoxides were measured as the primary oxidation products using a method adapted from Shanta and Decker (*20*). Emulsion (0.3 mL) was added to 1.5 mL of a mixture of isooctane/2-propanol (3:1) and vortexed three times for 10 s each, followed by centrifugation for 2 min at 10000*g*. The organic phase (0.2 mL or less according to the level of oxidation) was added to a mixture of methanol/butanol (2:1, v:v) followed by addition of 15 μL of 3.94 M thiocyanate and 15 μL of 0.072 M Fe^{2+}. The solution was vortexed, and after 20 min, the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

Secondary oxidation products were monitored with the thiobarbituric acid reactive substances (TBARS) method using a procedure described elsewhere (*21*). Briefly, at each incubation time, 1 mL of each sample was mixed with 2 mL of a TBA solution containing 20% trichloroacetic acid, 0.5% TBA, 0.2% EDTA, and 30 mM HCl in screw-capped tubes. Immediately afterward, 30 μL of 3% BHT in ethanol was added, and the tubes were then closed and vortexed. Samples were then heated in a boiling water bath for 15 min, cooled at room temperature, and centrifuged at 1600*g* for 20 min. The absorbance of the supernatant was measured at 532 nm using a Genesys 20 spectrophotometer (ThermoSpectronic). Concentrations were determined from a MDA standard curve produced from 1,1,3,3-tetrahydroxypropane.

The ability of the polysaccharides to scavenge peroxyl radicals was determined using a modified oxygen radical absorbance capacity (ORAC) assay (*22*). A 300 mM solution of AAPH in 75 mM potassium phosphate buffer at pH 7.0 was prepared for each experiment and kept on ice. Fluorescein sodium salt was dissolved to a concentration of 50 nM in phosphate buffer containing 0.1 mM EDTA before each set of experiments. For each run, fluorescein was equilibrated to 37 °C in a water bath for 15 min. Reagents were added in the order of polysaccharide (0, 0.02, 0.05, and 0.01 wt % in 75 mM phosphate buffer, pH 7.0), fluorescein, and AAPH at a final volume of 0.01, 2.7, and 0.02 mL, respectively. Fluorescence was recorded from 0 to 60 min every 10 min by taking 3 mL aliquots in which 40 μL of 500 mM ascorbic acid was added to stop the reaction followed by centrifugation for 10 min at 1750*g*. The fluorescence (excitation = 493 nm and emission = 515 nm; Hitachi F-2000 flourometer, Tokyo, Japan) of the supernatants was measured at 37 °C. Fluorescence was recorded every minute for 40 min. The relative radical absorbance capacity values of polysaccharides were expressed as μm of Trolox equivalents (TEs) per g of sample as determined from a Trolox standard curve over the range of 1.0−20 μM.

Iron-Binding Capacity. The ability of polysaccharides to bind iron was determined using a modified method of Debon et al (*23*). Dialysis tubing (Tube-O-Dialyzer, molecular mass cutoff 8 kDa, G-Bioscience, St. Louis, MO) was cleaned by heating it twice at 80 °C for 30 min in a 2% sodium bicarbonate and 1 mM EDTA solution. The tubing was then thoroughly rinsed with deionized water and stored at 5 °C in a 0.1% sodium azide solution. The polysaccharide solution (2.5 mL) was pipetted into the dialysis tubing and equilibrated for 30 min at room temperature in 100 mL of distilled water with constant stirring. One milliliter of a 20 mM Fe^{2+} solution (prepared from Fe_2SO_4 in 0.5 N HCl with 10 wt % of hydroxylamine hydrochloride) was then added to the flask so that the final concentration of ferrous outside the dialysis tube is 0.2 mM. After equilibrium for 12 h, a 5 mL aliquot from the outside the dialysis bag was mixed with 5 mL of ferrozine (5 mM in 100 mM Hepes buffer, pH 7.0)

and incubated for 30 min at room temperature, and the absorbance at 562 nm was measured using a Genesys 20 spectrophotometer (Thermo-Spectronic). The extent of iron binding was normalized with a reference where the polysaccharide solution inside of the dialysis bag was replaced by distilled water. The iron-binding capacity was expressed as % iron bound per g of polysaccharide, which was calculated as

$$(\text{Fe concentraton}_{\text{sample}} - \text{Fe concentraton}_{\text{reference}}) \times 100$$

Statistical Analysis. Duplicate experiments were performed with freshly prepared emulsion except for the chemical stability experiments, which were done three times. All data shown represent the mean values ± standard deviations of triplicate measurements. Statistical analysis of the effect of the polysaccharides on the ORAC and iron-binding capacity was performed using a one-way analysis of variance. A significance level of $p < 0.05$ between groups was accepted as being statistically difference. In all cases, comparisons of the means of the individual groups were performed using Duncan's multiple range tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Physical Stability of the Emulsions. Our preliminary experiments showed that, as expected, the polysaccharides (0−0.5 wt %) used in this study can negatively impact the physical stability of the oil-in-water emulsion. When the concentration of polysaccharide in emulsion system was higher than 0.15 wt %, creaming was observed to occur (data not shown). The creaming induced by high concentrations of polysaccharide is likely due to depletion flocculation (*24*). Because of this observed emulsion instability, all subsequent experiments were conducted with 0.1 wt % polysaccharide or less. Under these conditions, measurements of the volume mean particle diameter (d_{43}) of the emulsion system indicated that they were highly stable to aggregation in both the presence and the absence of polysaccharides with an average d_{43} value 0.32 μm, which did not change during the 10 days of storage regardless of polysaccharides type and concentrations ($p > 0.05$). The ζ-potential of all of the samples was approximately 0 mV at day 0, which is consistent for emulsions stabilized with nonionic surfactants. No major changes in ζ-potential were observed during storage (data not shown).

Prolonged storage at 15 °C showed no creaming after 6 months of storage except for the no polysaccharide control, which had a small amount of creaming as indicated by a serum layer at the bottom of the storage tubes. The excellent physical stability in the emulsions containing polysaccharides is likely due to the ability of the polysaccharide to increase the viscosity of the continuous phase, which decreases droplet collisions, thus decreasing flocculation and coalescence (*25*). In addition, viscosity enhancement by the polysaccharides would decrease creaming rates.

Effects of Polysaccharides on the Oxidative Stability of Oil-in-Water Emulsions. The ability of the different polysaccharides to inhibit lipid oxidation was evaluated by their ability to increase the lag phase of oxidation. In this study, lag phase was defined as the first data point statistically greater than the level of oxidation products at day 0. Both LM and HM pectin were able to inhibit lipid oxidation in the Brij-stabilized Menhaden oil-in-water emulsions as determined by both lipid hydroperoxides and TBARS (**Figures 1** and **2**). Importantly, both the LM and the HM pectins were able to inhibit lipid oxidation at concentrations where they did not cause the physical destabilization of the emulsion. The lag phase of lipid hydroperoxide and TBARS formation in the control (no polysaccharide) and 0.02 wt % LM pectin treatment was the same at 1 day of storage (**Figure 1A**,**B**). In the presence of 0.05 and 0.1 wt % LM pectin, the lag phase increased to 5 and 6 days for lipid hydroperoxides, respectively, and 4 days for TBARS for both pectin concentrations. For HM

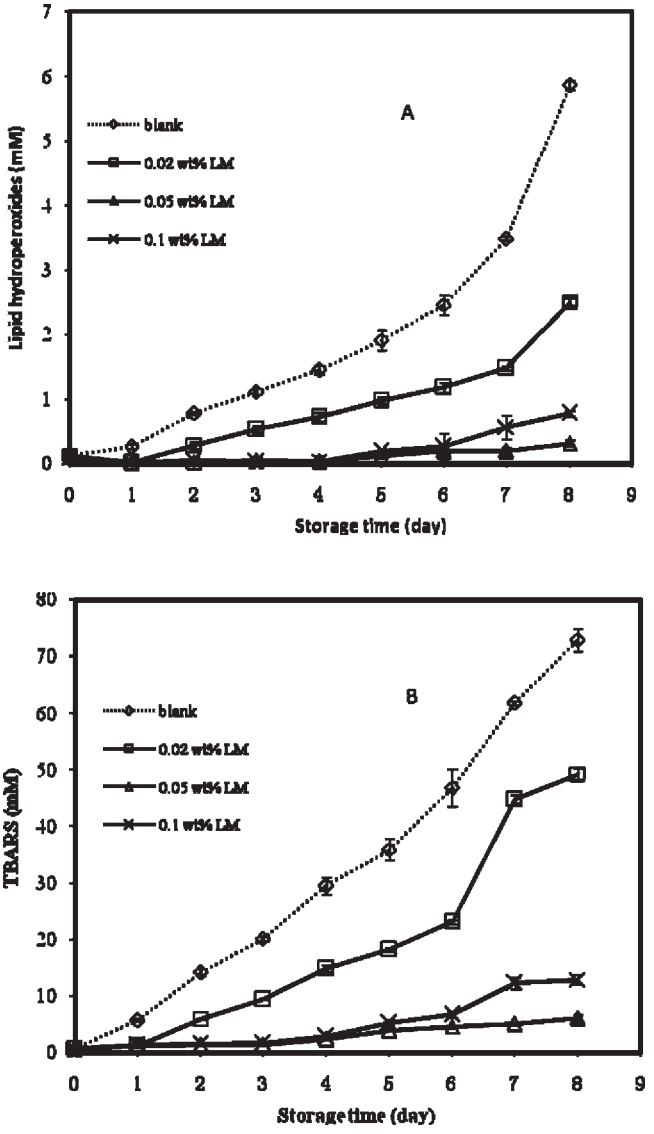

Figure 1. Formation of lipid hydroperoxides (**A**) and TBARS (**B**) in Menhaden oil-in-water emulsions (pH 7.0 and 15 °C) stabilized with Brij 35 in the presence of LM pectin (0−0.1 wt %). Data represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

pectin, the lag phase for the control and 0.02 wt % HM pectin was again the same at 1 day for both lipid hydroperoxides and TBARS (**Figure 2A**,**B**). Increasing the concentration of HM pectin to 0.05 wt % increased the lag phase for both lipid hydroperoxide and TBARS formation to 3 days, while the lag phase in the presence of 0.1 wt % HM pectin was 4 days for both lipid hydroperoxides and TBARS. LM pectin was more effective at inhibiting lipid oxidation than HM pectin. At a pectin concentration of 0.05 wt %, LM pectin increased lag phase from 1 (control) to 5 days for lipid hydroperoxides and 4 days for TBARS. In comparison, HM pectin at 0.05 wt % only increased the lipid hydroperoxide and TBARS lag phases to 3 days.

Unlike the pectins, carageenan did not consistently inhibit oxidation (**Figure 3A**,**B**). For 0.02 wt % carrageenan, lipid hydroperoxide concentrations remained low during the entire storage period. However, in the presence of 0.02 wt % carrageenan, TBARS were found to be the same as the control. These seemingly contradictory data are best explained as due to low concentration of carrageenan acting as a prooxidant. This could be due to chelation of iron by the carrageenan in a manner where iron is more reactive. The reactive chelated iron could then

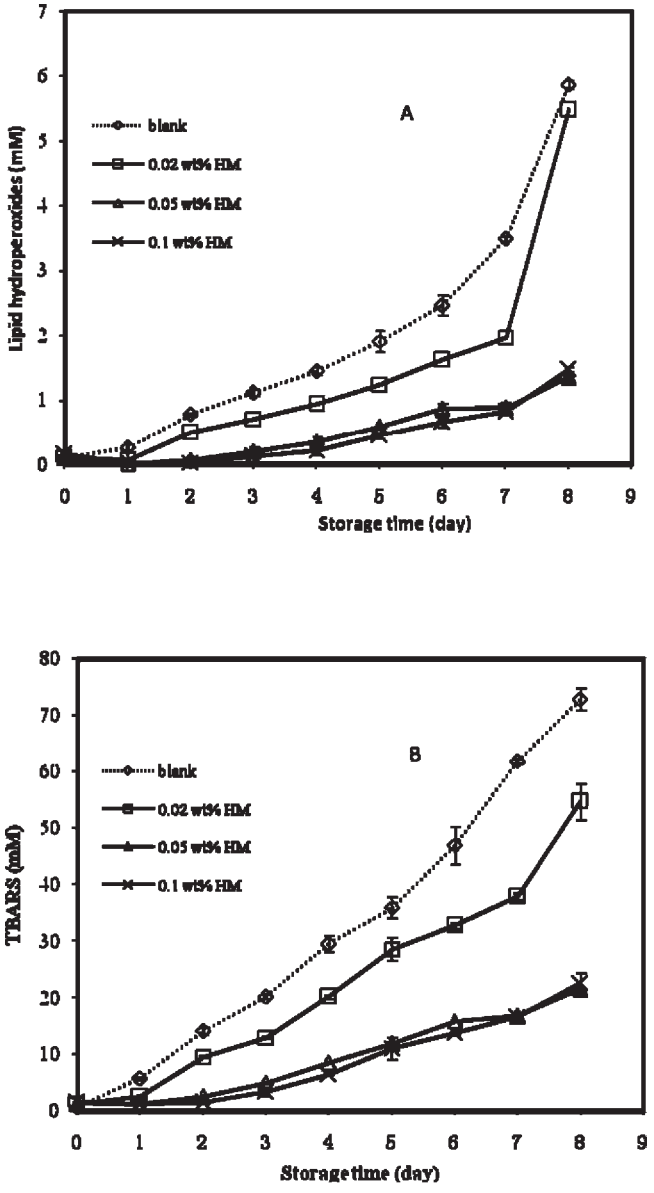

Figure 2. Formation of lipid hydroperoxides (**A**) and TBARS (**B**) in Menhaden oil-in-water emulsions (pH 7.0 and 15 °C) stabilized with Brij 35 plus HM pectin (0−0.1 wt %) in the continuous phase. Data represent means (n = 3) ± standard deviations. Some error bars are within data points.

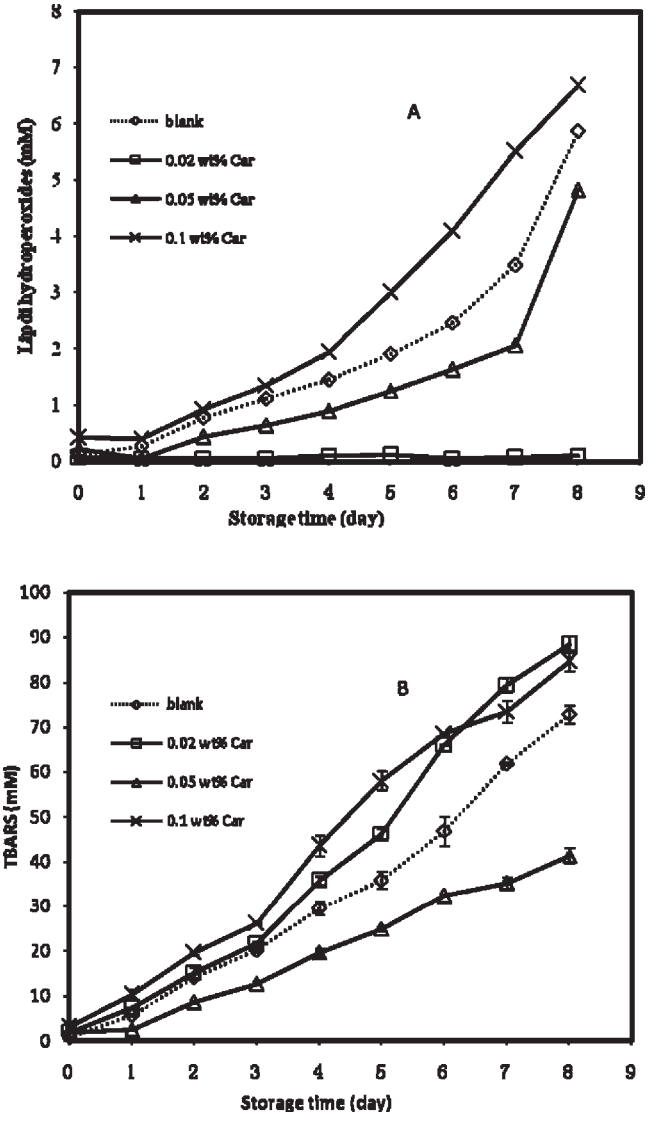

Figure 3. Formation of lipid hydroperoxides (**A**) and TBARS (**B**) in Menhaden oil-in-water emulsions (pH 7.0 and 15 °C) stabilized with Brij 35 plus α-carrageenan (car) (0−0.1 wt %) in the continuous phase. Data represent means (n = 3) ± standard deviations. Some error bars are within data points.

promote the decomposition of lipid hydroperoxides so that they did not accumulate but instead were converted to secondary lipid oxidation products, which were observed as high TBARS. Increasing carrageenan concentrations to 0.05 wt % did result in inhibition of both hydroperoxide and TBARS formation with a lag phase of 2 days for both. However, further increasing carrageenan concentrations to 0.1 wt % resulted in no inhibition of lipid hydroperoxides and TBARS formation as compared to the control. It is not clear why antioxidant activity was lost at higher concentrations. This trend could be due to the high concentrations of carrageenan increasing iron reactivity by increasing transition metal solubility or by the carrageenan acting as a reductant to convert transition metals to their more reactive states. Overall, it is clear that the carrageenan was not an effective and consistent antioxidant in the oil-in-water emulsion.

Alg was more effective than carrageenan but less effective than LM pectin (**Figure 4A**,**B**). At a concentration of 0.02%, Alg did not increase the lag phase of lipid hydroperoxide or TBARS formation as compared to the control. Increasing concentrations to 0.05 wt % increased the lag phase for lipid hydroperoxide and TBARS formation to 4 and 3 days, respectively. However, like carrageenan, increasing alginate to 0.1 wt % decreased its ability to inhibit hydroperoxide and TBARS formation with lag phases decreasing to 2 days for both lipid oxidation markers.

Radical-Scavenging Ability. One of the major pathways to inhibit lipid oxidation is by free radical scavenging (*26*). Polysaccharides have reducing sugars that could potentially inactivate free radicals. A common way to measure the ability of compounds to scavenge free radicals is with the ORAC assay, which uses water-soluble peroxyl radicals generated by AAPH. LM and HM pectin (**Table 1**) had peroxyl radical-scavenging activities over 15% higher than carrageenan and Alg. There is no significant difference peroxyl radical-scavenging activities between LM and HM pectin or between CA and SA ($P > 0.05$). However, it should be noted that the radical-scavenging activity of all of the anionic polysaccharides was very low with only scavenging peroxyl radicals to the same extent as < 2.07 μM Trolox.

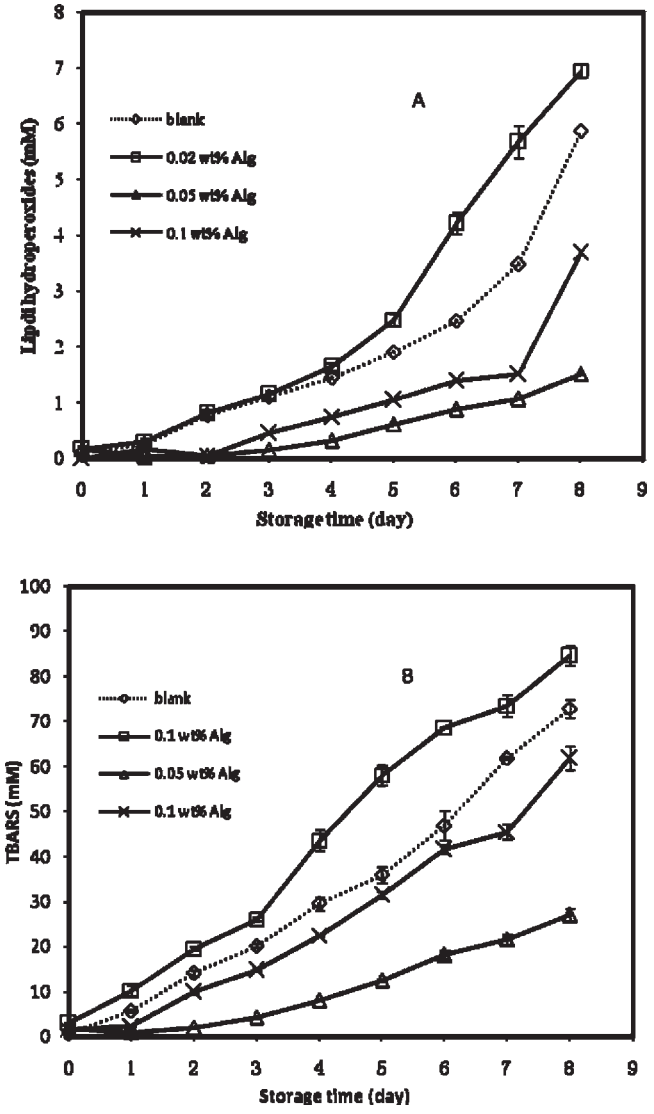

Figure 4. Formation of lipid hydroperoxides (**A**) and TBARS (**B**) in Menhaden oil-in-water emulsions (pH 7.0 and 15 °C) stabilized with Brij 35 plus Alg (0−0.1 wt %) in the continuous phase. Data represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

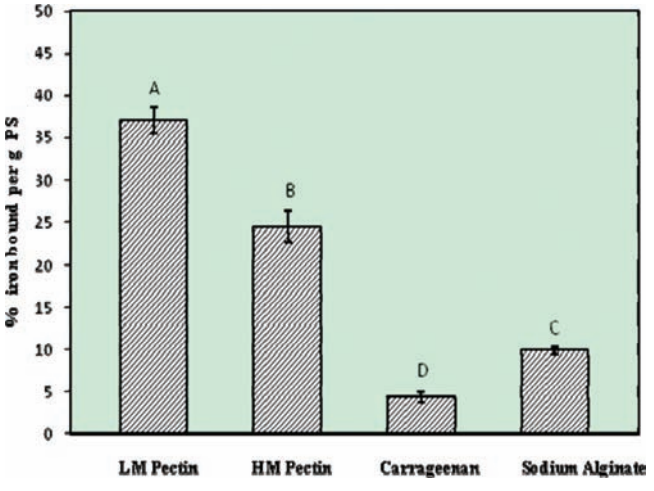

Figure 5. Percent iron bound/g polysaccharide during incubation of ferrous ion (0.2 mM) with various polysaccharides (0.1 wt %) for 24 h at pH 7.0. Data represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

Iron Ion-Binding Capacity. Polysaccharides can also inhibit lipid oxidation by chelating prooxidative metals (*26*). The ability of the polysaccharide to bind iron was tested in the presence of 0.10 wt % of each polysaccharide, the highest concentration used in the oil-in-water emulsion lipid oxidation experiments (**Figure 5**). As excepted, LM pectin bound more iron than HM pectin presumably due to its higher level of acid groups. Overall, the iron-binding capacity of the polysaccharides was in the order of LM pectin > HM pectin > Alg > carrageenan ($P < 0.05$).

CONCLUSIONS

Anionic polysaccharides have the potential to be used to inhibit lipid oxidation in oil-in-water emulsions if they are effective at concentrations that do not cause the physical destabilization of the emulsion. Therefore, in this study, concentrations of anionic polysaccharides were chosen that did not alter emulsion droplet charge or promote creaming. Anionic polysaccharides have also been shown to promoted lipid oxidation, especially if they are contaminated with transition metals. Therefore, in this study, the anionic polysaccharides were pretreated by Chelex-100, which is effective at binding and removing metals.

The current results indicate that HM and LM pectins can act as antioxidants in the continuous phase of an oil-in-water emulsion system. The major difference between the LM and the HM pectin used in this research is the level of galacturoinic acid esterification, which is 37 and 70%, respectively. LM pectin inhibited lipid oxidation in the Menhaden oil-in-water emulsions more effectively than HM pectin. The two pectins had similar free radical-scavenging activities so the observed difference in their ability to inhibit lipid oxidation is likely due to the higher iron-binding capacity of LM pectin.

LM and HM were more effective at consistently inhibiting lipid oxidation than Alg or carrageenan. Alg was slightly better at inhibiting lipid oxidation than carrageenan when added to the emulsions at 0.05%. This again is likely due to a higher iron-binding capacity of alginate as compared to carrageenan since the two polysaccharides had similar free radical-scavenging activities. However, upon increasing the concentrations of these two polysaccharides to 0.1%, their ability to inhibit lipid oxidation decreased. It is unclear why increasing polysaccharide concentrations increased lipid oxidation rates, but this could be due to the alginate and carrageenan binding transition metal to increase their solubility without decreasing their reactivity, or this could be due to the ability of alginate and carrageenan to reduce and convert transition metals to their more reactive states.

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