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Antioxidant Activity of a Proanthocyanidin-Rich Extract from Grape Seed in Whey Protein Isolate Stabilized Algae Oil-in-Water Emulsions

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Algae oil-in-water emulsions stabilized with 0.2% whey protein isolate (WPI) at pH 3.0 and 7.0 were chosen to evaluate antioxidant activity of a proanthocyanidin-rich extract from grape seed. In this emulsion system, (+)-catechin and ascorbic acid (620 μM) were found to be prooxidative at pH 3.0 and ineffective at pH 7.0. Grape seed extract was not able to effectively inhibit both lipid hydroperoxides and propanal formation when added to the emulsion at 124 μM. However, increasing the concentration of the grape seed extract to 620 μM resulted in inhibition of both lipid hydroperoxide and propanal formation at pH 3.0 and 7.0. None of the antioxidants tested had any effect on the physical stability of the WPI-stabilized emulsion. The superior antioxidant activity of the grape seed extract is likely due to the presence of oligomeric procyanidins which are better antioxidants compared to their monomeric counterparts.

KEYWORDS: Proanthocyanidin; grape seed extract; whey protein isolate; algae oil; antioxidant; emulsion; ω-3 fatty acids

INTRODUCTION

Surface-active proteins such as whey protein isolate (WPI) have been used as emulsifiers not only to form physically stable emulsions but also to increase the oxidative stability of oil-in-water emulsions (*1*, *2*). Surface-active proteins inhibit lipid oxidation through their ability to form a cationic emulsion droplet at pH values below the pI of the protein. Under these conditions, iron and other prooxidant metals are electrostatically repelled from the emulsion droplet surface, thus minimizing prooxidant metal−lipid interactions. The oxidative stability of WPI-stabilized oil-in-water emulsions can be further enhanced by the addition of metal chelators. Ethylenediaminetetraacetic acid (EDTA) has been shown to inhibit lipid oxidation as determined by reduction in both headspace propanal and lipid hydroperoxides in WPI-stabilized oil-in-water emulsions at pH 3.0 (*3*). However, EDTA is not a natural, label friendly food additive despite its use in the food industry. Thus, finding natural antioxidants that are effective in WPI-stabilized algae or fish oil-in-water emulsions at pH 3.0 is important to further enhance oxidative stability.

Algae oils produced by fermentation technologies have many benefits in functional foods due to their high ω−3 fatty acid content and their lack of environmental toxins. A typical ω−3 fatty acid content of commercially available algae oil is 3% eicosapentaenoic acid (EPA) and 46% docosahexaenoic acid (DHA) (data from Martek Biosciences Inc., Boulder, CO). Oils with such a high content of polyunsaturated fatty acids are easily oxidized, leading to rancidity and quality deterioration. On the other hand, recent studies that indicate dietary bioactive lipids promote good health have increased consumer demand for ω−3 fatty acid enhanced foods. The ω−3 fatty acids have been shown to be effective against coronary heart disease and type-2 diabetes and could be beneficial for pregnant and lactating women and their infants (*4*−*8*).

Therefore, developing efficient techniques and methods to protect ω−3 fatty acid enhanced foods from oxidation is needed before these functional foods can become a commercial reality.

The chemical composition of grape seed extracts (GSEs) has been gradually elucidated (*9*−*13*). The major antioxidants in GSE are proanthocyanidins, which are oligomers and polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin (*14*). GSE was found to be effective in scavenging free radicals and inhibiting lipid oxidation in model systems including H_2O_2/NaOH/DMSO (*15*), hereditary cataractous rats (*16*), lecithin liposomes (*17*), and low-density lipoprotein (*18*). Since grape seed extract has been shown to be an effective antioxidant and since the FDA recently categorized GSE as a generally recognized as safe (GRAS; *19*) food ingredient, this research was designed to test if GSE could inhibit lipid oxidation in a WPI-stabilized algae oil-in-water emulsion at pH 3.0 and pH 7.0. The antioxidant activity of GSE was compared to that of other water-soluble antioxidants including ascorbic acid (AA) and (+)-catechin.

MATERIALS AND METHODS

Materials. Algae oil was obtained from Martek Biosciences, Inc., Boulder, CO. (+)-Catechin, ascorbic acid, imidazole, sodium acetate, ferrous sulfate, cumene hydroperoxide, and propanal were purchased

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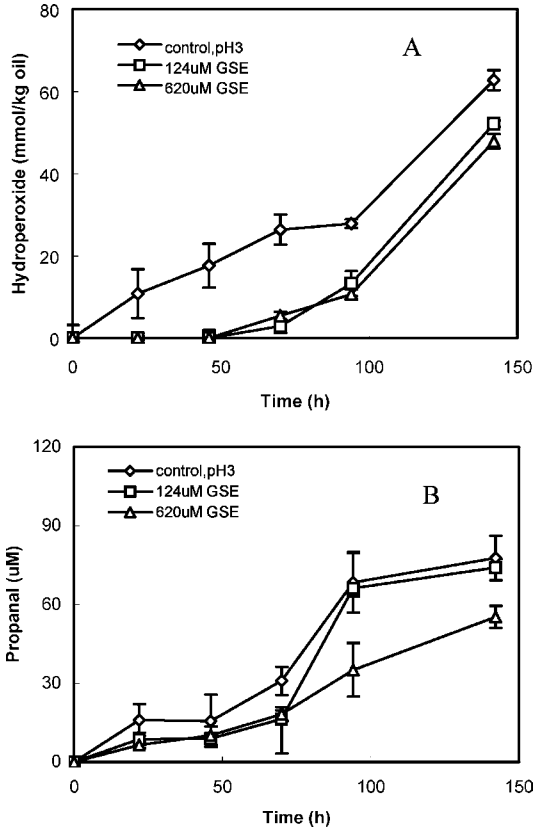

Figure 1. Effect of GSE on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 3 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

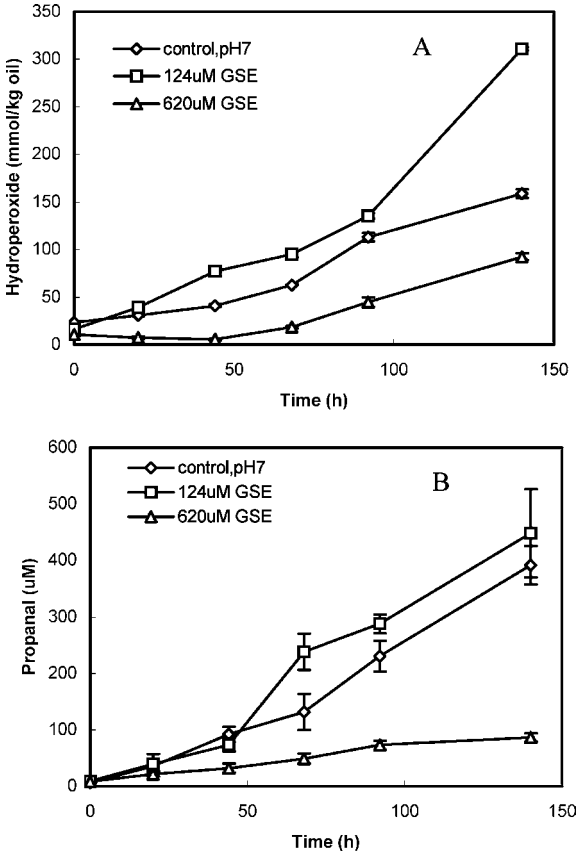

Figure 2. Effect of GSE on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 7 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

from Sigma Chemical Co. (St. Louis, MO). Grape seed extract (Gravinol-S, which contains 38% proanthocyanidins as reported by the manufacturer) was obtained from Kikkoman Corp. (Biochemicals Division, Noda City, Japan). WPI was obtained from Davisco Food International Inc. (Eden Prairie, MN). Proteins were used without further purification. The protein content of WPI was 97.6%. The major protein components of WPI were 55−61% β-lactoglobulin, 19−22% α-lactalbumin, and 6−8% bovine serum albumin. All other reagents were of analytical grade or purer.

Preparation and Characterization of Emulsions. An oil-in-water emulsion was prepared using 5.0 wt % algae oil and 95% acetate−imidizole buffer (5 mM each, pH 3.0 or 7.0) containing 0.2 or 0.5% WPI. Oil-in-water emulsions were made by blending the lipid and aqueous phases for 2 min using a hand-held homogenizer (M 133/1281, Biospec Products, Inc., Bartlesville, OK.). The coarse emulsion was then homogenized three times at 5000 psi through a high-pressure valve, two-stage APV Lab 1000 homogenizer (Albertslund, Denmark). Stock solutions of antioxidants were added separately to freshly prepared emulsions. The particle size distribution ($d_{3,2}$) of the emulsions was measured using a Coulter LS 230 laser light scattering instrument (Coulter Corp., Miami, FL). Droplet size distributions were measured after homogenization to monitor emulsion stability. The range of $d_{3,2}$ of the emulsions was from 0.72 to 0.102 μm. Emulsion droplet charge (zeta potential, ξ) was measured by directly injecting diluted (1:1000) oil-in-water emulsions into the measurement chamber of a ZEM 5003 Zetamaster (Malvern Instruments, Worchester, U.K.). The ξ-potential measurements are reported as the average of two separate injections, with five readings made per injection. The range of zeta potential of the emulsions used in these experiments was from −56.0 to −60.3 mV.

Measurement of Lipid Oxidation. To monitor lipid hydroperoxide formation during storage, emulsions (5 mL) were placed in lightly sealed screw cap test tubes and allowed to oxidize at 37 °C in the dark. Lipid hydroperoxides were measured using a modified method of Shantha and Decker (*20*) by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, 3 times) and isolation of the organic solvent phase by centrifugation at 1000*g* for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4 \cdot 7\ H_2O$). The absorbance of the solution was measured at 510 nm, 20 min after addition of the iron. Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide (0−300 μM).

For headspace analysis, emulsion samples (1 mL) were placed into 10 mL headspace vials and sealed with poly(tetrafluoroethylene) butyl rubber septa. Headspace propanal was determined using a Shimadzu 17A gas chromatograph equipped with a Hewlett-Packard 19395A headspace sampler (*21*). The headspace conditions were as follows: sample temperature, 40 °C; sample loop and transfer line temperature, 110 °C; pressurization, 10 s; venting, 10 s; injection, 1.0 min. The aldehydes were separated isothermally at 70 °C on a 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 (0−750 μM) dissolved in WPI solution.

Measurement of Total Phenolics. Total phenolics in the grape seed extract were analyzed according to the method of Zoecklin and coworkers (*22*) using gallic acid as a standard. Values were reported as gallic acid equivalents (GAE) and were expressed using units of μM.

Statistical Analysis. Assays were measured in triplicate. Statistical analysis was performed using Student's *t* test (*23*).

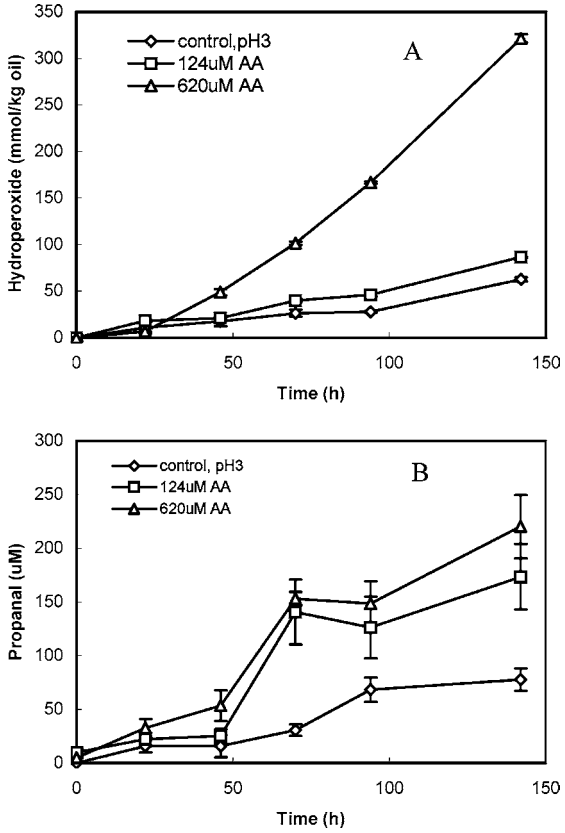

Figure 3. Effect of AA on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 3 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

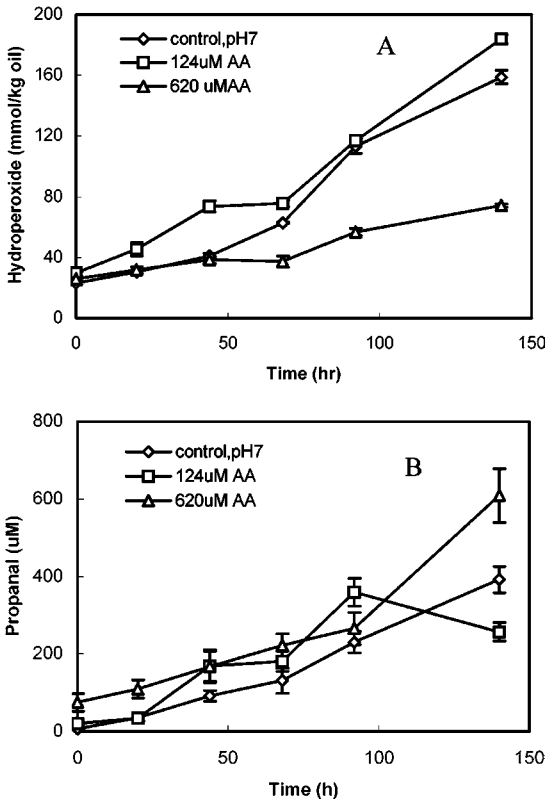

Figure 4. Effect of AA on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 7 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

RESULTS

GSE was added to the WPI-stabilized algae oil-in-water emulsion at concentrations of 100 and 500 ppm that represent 124 and 620 μM gallic acid equivalents, respectively. These concentrations of GSE were chosen because levels higher than 500 ppm can produce astringent flavors in foods. Ascorbic acid is one of the major water-soluble antioxidants used in foods, so its activity was also evaluated in the WPI-stabilized algae oil-in-water emulsions. (+)-Catechin was evaluated because it is a common monomer found in procyanidins. At pH 3.0, both GSE concentrations (124 and 620 μM) were able to decrease ($p \leq 0.05$) lipid hydroperoxide concentrations during the entire storage period (**Figure 1A**). Treatments containing 124 μM GSE had similar propanal concentrations compared with the control during the entire storage period (**Figure 1B**). A higher concentration of GSE (620 μM) was able to significantly ($p \leq 0.05$) decrease propanal formation in the emulsion at pH 3.0 after 48 h of incubation (**Figure 1B**). At pH 7.0, 124 μM of GSE increased lipid hydroperoxide concentrations after 24 h of incubation and increased propanal concentrations after 68 h of incubation. The higher concentration of GSE (620 μM) was able to inhibit both lipid hydroperoxide and propanal formation in the emulsion at pH 7.0 during the entire incubation period (**Figure 2**).

Ascorbic acid at 124 and 620 μM was prooxidative at pH 3.0 (**Figure 3**). Lipid hydroperoxide was slightly increased by 124 μM after 72 h, and headspace propanal increased after 48 h of storage. The higher concentration of ascorbate was even more prooxidative, causing an increase in both hydroperoxides and propanal after 24 h of incubation (**Figure 3**). At pH 7.0, 124 μm ascorbate caused an increase in hydroperoxide while 620 μM was able to inhibit hydroperoxide formation after 48 h of storage (**Figure 4A**). Propanal concentrations were similar for the control and 124 μM ascorbate, while 620 μM ascorbate increased propanal levels, being higher than the control at all times except 92 h (**Figure 4B**).

In a comparison of antioxidants at 620 μM in the WPI-stabilized algae oil-in-water emulsion at pH 3.0, GSE again decreased lipid hydroperoxides and propanal while ascorbate increased both of the lipid oxidation markers (**Figure 5**). (+)-Catechin was found to be prooxidative, increasing hydroperoxides after 72 h and propanal after 48 h. At pH 7.0 (**Figure 6**), GSE again decreased both hydroperoxides and propanal while ascorbate decreased hydroperoxides but increased propanal. (+)-Catechin decreased hydroperoxide concentrations after 24 h. (+)-Catechin increased propanal concentrations during the first 72 h of incubation.

None of the antioxidants tested altered emulsion droplet size or the surface charge of the droplets as measured by zeta potential (results not shown). In addition, the emulsions did not exhibit creaming as determined by visual observation. These data suggest that the antioxidants did not alter the physical stability of the emulsions.

DISCUSSION

In some cases, an antioxidant can decrease the concentration of lipid hydroperoxides while not being able to influence the concentrations of secondary lipid oxidation products. A possible scenario for this observation is the ability of the antioxidant to reduce transition metals to a more prooxidative state. Reduced transition metals can decompose lipid hydroperoxides with the formation of the oxidized state of the metal. Following hydroperoxide decomposition, the resulting alkoxyl radical can

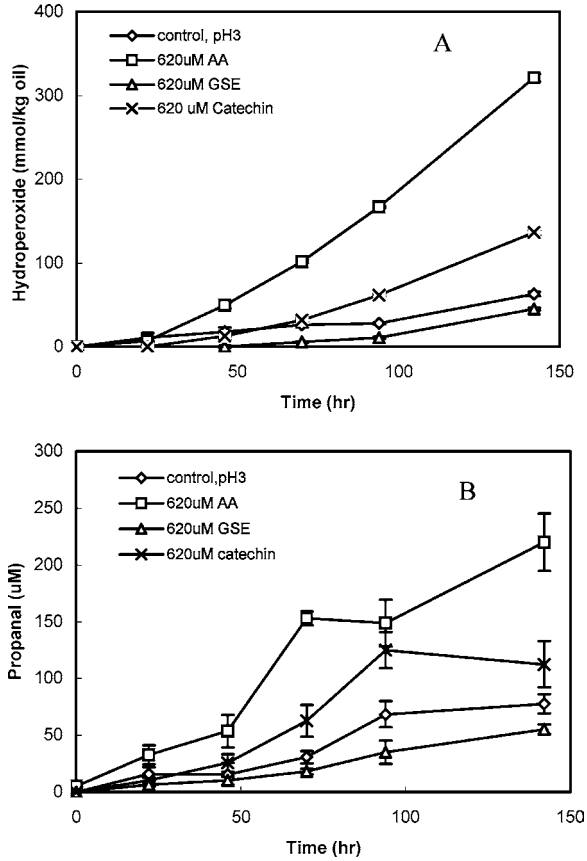

Figure 5. Effect of AA, GSE, and (+)-catechin on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 3 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

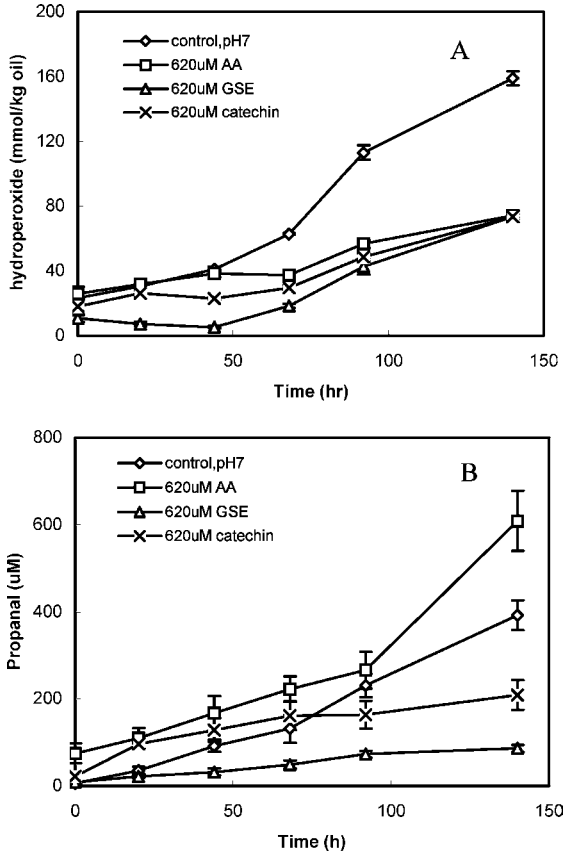

Figure 6. Effect of AA, GSE, and (+)-catechin on the formation of lipid hydroperoxide (**A**) and headspace propanal (**B**) in algae oil-in-water emulsions stabilized by 0.2% whey protein isolate at pH 7 at 37 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within data points.

undergo β-scission reactions that produce volatile secondary lipid oxidation products. Therefore, antioxidants that can promote metal reduction can alter the formation of lipid oxidation markers such that hydroperoxides do not increase (due to their decomposition) while increasing formation of secondary lipid oxidation products (e.g., propanal). In order for an antioxidant to be effective, it must be able to inhibit the formation of volatile secondary lipid oxidation products that are perceived as rancidity. Interactions between antioxidants and transition metals could be very important since prooxidant metals are thought to promote lipid oxidation in oil-in-water emulsions (*24*). The ability of an antioxidant to either inhibit or promote oxidation can be very concentration dependent. This would be true of an antioxidant that can reduce prooxidant metals where at low concentrations it can be prooxidative due to metal reduction and at high concentrations it can be antioxidative when enough radical scavenging capacity is present to overcome the prooxidant nature of the compound.

Of all of the antioxidants tested, GSE showed the most promise as an antioxidant in the WPI-stabilized algae oil-in-water emulsion. While the effectiveness of the low concentration of GSE (124 μM) was inconsistent, the higher concentration (620 μM) was found to inhibit both hydroperoxide and propanal formation at both pH 3.0 and 7.0. This is in contrast to the other antioxidants whose activity was inconsistent. Both ascorbate and catechin were prooxidative at pH 3.0 and ineffective at pH 7.0 since they were unable to inhibit both hydroperoxide and propanal formation. The prooxidant activity of ascorbate and catechin can be explained by their ability to reduce transition metals to increase prooxidant activity (*25*−*27*). The increased prooxidant activity of ascorbate and catechin at pH 3.0 compared to 7.0 could be due to the fact that iron is more soluble at low pH meaning that more iron could be available to interact with the reducing components.

As mentioned previously, grape seed extract contains procyanidins (36%) that are oligomers of (+)-catechin or (+)-epicatechin. The ability of GSE to inhibit oxidative reactions more effectively than ascorbate and catechin has also been observed in a model system containing linoleic acid and β-carotene in 50% ethanol (*28*). The superior antioxidant activity of the GSE could be due to the fact that the oligomeric procyanidins are more effective than their monomer counterparts (*29*). In addition, high molecular weight procyanidins are effective at inhibiting lipid oxidation promoted by lipid-soluble peroxyl radicals, suggesting that the larger oligomers of (+)-catechin and (−)-epicatechin may be lipid-soluble or surface active. Since nonpolar antioxidants that are retained in the droplets of oil-in-water emulsions are more effective than polar antioxidants (*30*), this could also help explain the superior antioxidant activity of GSE. An additional reason why the GSE was effective in the WPI-stabilized algae oil system could be through its ability to bind to protein. Sarni-Manchado et al. (*30*) found that the ability of grape seed procyanidins to bind to proteins increased with increasing molecular weight. The ability of high molecular weight procyanidins to bind to proteins may have resulted in increased association of antioxidants at the surface of the WPI-stabilized emulsion droplets. This would effectively increase the concentration of antioxidants at a

location where many of the oxidative reactions are occurring, thus potentially increasing the ability of the GSE to inhibit lipid oxidation.

In conclusion, this research indicates that GSE could be an effective antioxidant in oil-in-water emulsions stabilized by whey protein isolate. Since GSE is a natural product that was recently recognized as GRAS, it represents a new natural antioxidant that could be effective at protecting bioactive lipids in foods.

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