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ORIGINAL PAPER

Antioxidant Effects of Berry Phenolics Incorporated in Oil-in-Water Emulsions with Continuous Phase β-Lactoglobulin

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Abstract The purpose of this study was to evaluate the effects of berry phenolics, in this case, black currant (*Ribes nigrum*) anthocyanins and raspberry (*Rubus idaeus*) ellagitannins, in the presence of continuous phase β-lactoglobulin (β-Lg), on the oxidative stability of Brij 35-stabilized corn oil-in-water emulsions. The extent of lipid oxidation in emulsions was measured by determining the formation of lipid hydroperoxides and hexanal, and extent of protein oxidation by monitoring the loss of tryptophan and cysteine residues in the continuous phase β-Lg. Berry phenolics at concentration levels of 20 and 50 μM were able to prevent lipid oxidation with and without β-Lg in the aqueous phase. The results show that a combination of β-Lg and berry phenolics was more efficient in inhibiting hexanal formation than either component alone. Synergistic effects on antioxidant activity toward hexanal were observed only at the 20 μM levels of berry phenolics in combination with continuous phase β-Lg. The berry phenolics were also able to inhibit the oxidation of tryptophan and cysteine residues of β-Lg. The results show that the amino acid residues were oxidized prior to the propagation of lipid oxidation. This suggests that these amino acids were able to inhibit fatty acid scission. The information gained from this study would be useful in protecting emulsion-based food products from oxidative deterioration.

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Introduction

Many foods are found in the form of emulsions, either as *oil-in-water* (e.g. milk and cream) or *water-in-oil* (e.g. margarine and butter). An emulsion is a mixture of two or more immiscible liquids, where one liquid (the dispersed phase) is dispersed in the other (the continuous phase). The oxidative and physical stability of emulsions depends on multiple factors such as the type of ingredients (lipids, emulsifiers, antioxidants, minerals, flavors, colorants, etc.), pH and ionic strength, presence of oxygen, processing and storage conditions [1]. In an oil-in-water emulsion, the susceptibility of lipids to oxidation is one of the main reasons for quality deterioration [2]. Moreover, free radicals derived from lipid oxidation reactions are easily transferred to other molecules such as proteins, carbohydrates and vitamins, especially in the presence of metals [3–5]. The oxidative attacks on food macromolecules contribute to deterioration of flavor, aroma, color (browning reactions), and nutritive value. Protein oxidation leads to loss of amino acids and solubility, changes in texture, alterations in protein functionality and may even lead to toxic products [6, 7].

In order to reduce and control lipid and protein oxidation, antioxidant compounds can be added to emulsions. Plant materials rich in phenolic compounds have gained much attention because they exhibit a wide range of activities such as antioxidant, antimicrobial, antimutagenic as well as anti-inflammatory activities [8–10]. Phenolic compounds act as antioxidants by donating electrons and terminating radical chain reactions [11], as well as

chelators by binding metal ions [12]. The antioxidant role of natural plant materials rich in phenolics such as extracts from berries [13, 14], green tea [15], raisins [16], olives [17], grape seeds [18], and cactus pear fruits [19] have been tested in a variety of oil-in-water emulsions. Research by Viljanen et al. [13, 14] reported that anthocyanins isolated from black currant, raspberry and lingonberry as well as raspberry and blackberry juices showed protection toward lipid and protein oxidation in whey-protein-stabilized rapeseed oil-in-water emulsion. In another study, the antioxidant activity of cactus pear fruits toward lipid oxidation was evaluated in Tween80-stabilized fish oil-in-water emulsions [19]. Olive polyphenols in Tween20-stabilized olive oil-in water emulsions [17], raisin extract in Tween20-stabilized corn oil-in-water emulsions [16], and proanthocyanidin-rich grape extract in whey protein-isolate-stabilized algae oil-in-water emulsions [18] were shown to exhibit antioxidant activity toward lipid oxidation.

Another antioxidant approach is to use proteins and peptides in oil-in-water emulsions to prevent oxidative reactions. This could be done in the form of emulsifiers or as ingredients added to the aqueous phase. Sodium caseinate with lactose [20], casein hydrolysates [21], and whey proteins [22] as well as β-lactoglobulin (β-Lg) [23] have been found to increase the stability of oil-in-water emulsions. Soy protein isolate, sodium caseinate and whey protein isolate in the continuous phase or as emulsifiers can enhance the oxidative stability of oil-in-water emulsions [24, 25]. In addition, native, heat-treated and enzymatic hydrolysates of β-Lg in the continuous phase of menhaden oil-in-water emulsions have been shown to inhibit lipid oxidation reactions [26–28]. These studies showed that heat-treated and enzymatic hydrolysates of β-Lg were more potent in inhibiting lipid oxidation than the native protein due to increased free radical scavenging and metal chelating activity. Few studies have established the effects of proteins on the total antioxidant activity of different phenolic compounds in oil-in-water emulsions. Proteins such as BSA and ovalbumin have been shown to increase the stability of oil-in-water emulsions in the presence of phenolic antioxidants even though BSA itself does not act as an antioxidant [15, 29].

While numerous reports on the activity of antioxidants in oil-in-water emulsions are found in the literature, very few studies have been published on the effects of phenolic antioxidants on protein oxidation. Although it has been shown in the aforementioned studies that continuous phase proteins can improve the stability of oil-in-water emulsions, there are no previous studies on the roles of phenolic compounds in the presence of continuous phase protein on the oxidative stability of oil-in-water emulsions. Therefore, the aim of this study was to investigate the effects of berry phenolics such as black currant anthocyanins (*Ribes nigrum*) (BCA) and raspberry ellagitannins (*Rubus idaeus*) (RE) and continuous phase β-Lg on lipid and protein oxidation reactions in corn oil-in-water emulsions. Heat treated β-Lg was chosen due to its earlier proposed ability to inhibit oxidation reactions. Understanding the activities of phenolic compounds and proteins could offer a foundation for their more systematic use in the food industry as functional ingredients, and thereby protect the dispersed food products from oxidative deterioration.

Materials and Methods

Materials

Corn oil (Mazola®) was purchased from a local grocery store. According to the manufacturer it contained 14.4 mg/100 g of vitamin E. Food-grade lyophilized β-Lg was donated by Davisco Foods International Inc. (Eden Prairie, MN). All other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Distilled, deionized water was used throughout the study. Fresh raspberries (*R. idaeus*) and black currants (*R. nigrum*) were purchased from a local market in Finland. The berry samples were immediately vacuum packed and stored in a freezer at −20 °C until use.

Isolation of Berry Phenolics

Berry samples were freeze-dried prior to analysis and stored at −20 °C until use. Extraction and isolation of BCA and RE was carried out as described by Kähkönen et al. [30]. The berry phenolic fractions were further purified by preparative HPLC and the interfering sugars were removed by solid phase extraction (SPE) as described by Kähkönen and co-workers [30]. Berry isolates were freeze-dried and stored at −20 °C.

Phenolic Profiles of Berries

The HPLC analysis of phenolics was performed according to the methods of Koski et al. [31] and Kähkönen et al. [8]. Catechin, chlorogenic acid, cyanidin-3-glucoside, ellagic acid, gallic acid, procyanidin B1, rutin, and sinapic acid were used as external standard compounds. The phenolic profiles are shown in Table 1.

Preparation of Heat-Treated β-Lactoglobulin

β-Lactoglobulin (1 mg/mL) in Tris–HCl buffer (pH 8.0) containing sodium azide (0.02% w/w) as an antimicrobial

Table 1 Phenolic composition of berry isolates (mg/g, means ± SD)

Compound	Black currant anthocyanins (mg/g)	Raspberry ellagitannins (mg/g)
Anthocyanins	314 ± 44	18 ± 0
Ellagitannins	ND	369 ± 44
Ellagic acid	ND	16 ± 2
Flavanols	12 ± 1	ND
Flavonols	ND	8 ± 1

ND Not detected

agent, was incubated at 37 °C for 24 h [32]. Following incubation, the protein was heated at 95 °C in a water bath for 15 min, followed by cooling in a water bath at ambient temperature for 10 min. After these treatments were completed, β-Lg was lyophilized and stored at −80 °C until use.

Preparation of Emulsions

Brij 35 (polyoxyethylene lauryl ether)-stabilized 5 wt% corn oil-in-water emulsions were used in all oxidation studies. The emulsifier solution was prepared by dispersing (0.5 wt%, i.e. 4.2 mM) Brij 35 in 10 mM sodium phosphate buffer (SPB) (pH 7.0) containing 0.02 wt% sodium azide as an antimicrobial agent, and stirring for 1 h at ambient temperature. Corn oil was added to the aqueous emulsifier solution and homogenized at high speed (20,000 min^{-1}) for 2 min with a homogenizer (Telmar® Tissumizer, Germany) to produce a coarse 5% (w/w) oil-in-water emulsion. Emulsion droplet size was then further reduced with an ultrasonicator (Fisher Sonic Dismembrator 500, Pittsburgh, PA) at 4 °C for 2 min at 70% power and 0.5 duty cycle. After preparation of the corn oil-in-water emulsions, β-Lg (0.5 mg/g oil in emulsion, i.e. 25 μg/100 g emulsion) was dissolved in the freshly made corn oil-in-water emulsions by stirring. Control sample without β-Lg was also prepared.

Addition of berry phenolics into the corn oil-in-water emulsions was made as follows: first, stock solutions of BCA and RE in methanol (2 mM) were prepared. The concentrations of BCA and RE were calculated as cyanidin-3-glucoside and ellagic acid equivalents, respectively, because they represented the main phenolic groups present in the isolates. These calculations were made according to the method by Kähkönen et al. [8]. However, as this calculation method does not reflect the wide range of phenolics with different molecular weights present in the isolates, the concentrations of berry extracts are also expressed as mg/mL of stock solution. Thus, the concentrations of BCA and RE extracts in the stock solutions were 3.1 and 2.0 mg/mL, respectively. Second, the BCA and RE stock solutions (1 or 2.5 mL corresponding to 20 or 50 μM final concentration, respectively) were first pipetted into empty Erlenmeyer flasks, and evaporated to dryness under nitrogen flow. Then, the prepared corn oil-in-water emulsion (100 mL) with β-Lg or without β-Lg was added into the flasks containing the dried extract and stirred. Control samples without berry phenolics were also prepared. All glassware used for the emulsion preparation and sample storage was acid washed with 2.0 N HCl to remove residual metals.

Physical Stability of Emulsions

The particle size distribution of the emulsions was determined by a laser diffraction instrument (Malvern Mastersizer, Malvern Instruments, Worcestershire, UK). In laser diffraction, particle size distributions are calculated by comparing the sample's scattering pattern with an appropriate optical model using a scattering theory (i.e. Mie theory). Results are given as surface area mean diameter: $d_{32} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$ and volume mean diameter: $d_{43} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of droplets of diameter d_i. The mean particle sizes of the emulsions ranged from 0.45 to 0.46 μm (d_{32}), and 0.94 to 0.96 μm (d_{43}) showing monomodal distributions. The particle size distributions did not change during the course of the experiment.

Lipid Oxidation

All corn oil-in-water emulsion samples were allowed to oxidize in the absence of light at 55 °C for up to 24 days. Lipid oxidation was monitored by following the formation of lipid hydroperoxides and hexanal. Lipid hydroperoxides were determined according to a method of Nuchi et al. [33] by mixing 0.3 mL of emulsion with 1.5 mL 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 (100 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v) followed by addition of thiocyanate/ferrous solution (30 μL) (prepared by mixing equal volumes of 0.132 M $BaCl_2$ with 0.144 M $FeSO_4$, centrifuging, and mixing equal volumes of the clear ferrous solution with 3.97 M ammonium thiocyanate). The absorbance of the solution was measured 20 min after the addition of the iron at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA) [34]. Hydroperoxide concentrations were determined using an external standard curve made from cumene hydroperoxide.

The formation of secondary oxidation products measured as hexanal was determined by placing 1 mL of the emulsion samples into 10-mL headspace vials sealed with poly(tetrafluoroethylene)/silicone septa. Headspace hexanal was

detected using a gas chromatograph (Shimadzu 17A) equipped with an AOC-5000 headspace autosampler (Shimadzu, Kyoto, Japan). A 30 m × 0.32 mm Equity DB-1 column (Supelco, Bellefonte, PA) with a 1-μm film thickness was used for separations. Samples were heated to 55 °C in the autosampler heating block and held there for 13 min. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle (Supelco) was injected into the sample vial for 1 min to absorb volatiles and then injected into the 250 °C injector port for 3 min. The gas chromatograph ran for 10 min at 65 °C for each sample. Helium was used as the carrier gas, with a total flow rate of 15.0 mL/min. A flame ionization detector at a temperature of 250 °C was used. Hexanal concentrations were determined from peak areas using a standard curve made from hexanal.

The percent inhibition (i.e. antioxidant activity) of plant materials against formation of lipid hydroperoxides and hexanal were calculated for day 24: $(C_0 - C_1)/C_0 \times 100$, where C_0 was the concentration of lipid hydroperoxides or hexanal in control sample and C_1 was the lipid hydroperoxides or hexanal in antioxidant sample. The inhibitions were expressed as percentages.

Amino Acid Oxidation

All amino acid oxidation assays were performed on continuous phase proteins that were free of Brij-stabilized oil droplets. To remove oil droplets, emulsions were centrifuged (1,000*g*) for 30 min in Amicon Ultra centrifugal tubes with regenerated cellulose membranes (MWCO 100000) (Millipore, Billerica, MA). Separation of the protein from the berry phenolics was performed chromatographically. The clear sample after the ultracentrifugation was passed through an Econo-Pac® 10 DG-desalting column with exclusion limit at 6,000 Da (Bio-Rad, Richmond, CA) equilibrated with SPB (pH 7.0). The protein was separated from phenolics by elution with the buffer, and the protein was collected. A protein assay (Bradford—Coomassie Blue, Pierce, Thermo Fisher Scientific Inc., Rockford, IL) was used to adjust protein to the same concentrations. Tryptophan residues were measured directly by fluorescence spectroscopy (Photon Technology Intl. Inc., Birmingham, NJ) with excitation at 283 nm and emission at 331 nm. Cysteine residues were measured directly using a sensitive fluorescent probe 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) as was described by Carr et al. [35]. First, protein was denaturated with 8 M urea at room temperature for 1 h to expose the buried cysteine residues in *β*-Lg, and to measure total sulfhydryls. Then, 100 mM SPB, pH 8.0, containing 1 mM diethylenetriaminepentaacetic acid (300 μL) was added to the mixture. ABD-F (10 μL of a 10 mM stock solution in buffer) was added to the protein solution, mixed by vortexing, and incubated in a 60 °C water bath for 20 min. Derivatized cysteine was measured by fluorescence at an excitation wavelength at 365 nm and emission at 492 nm. Concentrations were expressed relative to day 0 for tryptophan and cysteine concentrations because differences in their concentrations occurred in the different emulsions.

Statistical Analysis

All experiments were performed on triplicate samples. Statistical analysis was performed using a mixed procedure (proc mix) by SAS 9.1 (SAS Institute Inc., Cary, NC). Significant interactions where sliced in order to test the significance at each level of treatment.

Results and Discussion

Lipid Oxidation

The lipid oxidation reactions were investigated in emulsions containing the following: (a) berry phenolics, (b) continuous phase *β*-Lg, (c) berry phenolics and continuous phase *β*-Lg, or (d) none of the above (i.e. control). The formation of primary lipid oxidation products, i.e. lipid hydroperoxides, was measured in Brij 35-stabilized corn oil-in-water emulsions (pH 7) with berry phenolics and/or *β*-Lg at 55 °C over 24 days (Figs. 1, 2). In this study, the lag phase of lipid hydroperoxides was similar for all corn oil-in-water emulsions with or without continuous *β*-Lg (0.5 mg/g oil) (Fig. 1). The duration of the initial lag phase is most likely due to natural tocopherols present in corn oil acting as free radical scavengers. The ability of

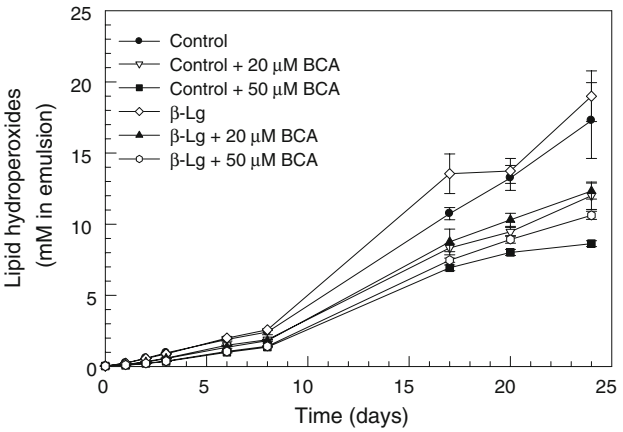

Fig. 1 Formation of lipid hydroperoxides in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with and without continuous phase *β*-lactoglobulin (*β-Lg*, 0.5 mg/g oil) with added black currant anthocyanins (*BCA*) during oxidation at 55 °C in the dark (means ± SD, $n = 3$)

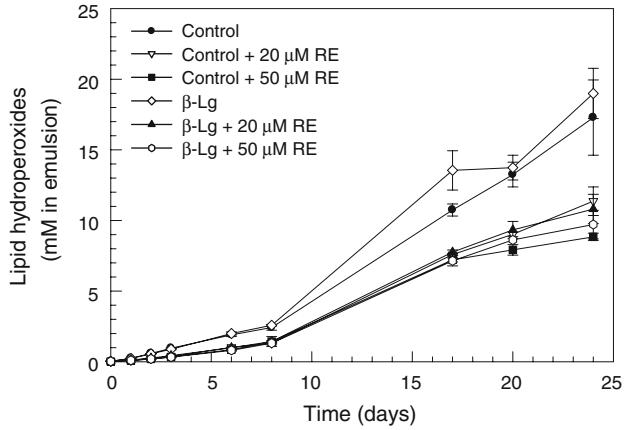

Fig. 2 Formation of lipid hydroperoxides in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with and without continuous phase *β-lactoglobulin* (*β-Lg*, 0.5 mg/g oil) with added raspberry ellagitannins (*RE*) during oxidation at 55 °C in the dark (means ± SD, $n = 3$)

α-tocopherol to act as chain-breaking antioxidant scavenging lipid peroxyl radicals by donating hydrogen to a lipid peroxyl radical that otherwise would propagate the radical chain reaction of lipid peroxidation has been shown in LDL [36, 37]. Although other studies of oil-in-water emulsions have used oils stripped of tocopherols [13, 18, 19], this study used corn oil with natural tocopherols, which is more relevant to normal food formulation and processing used in the food industry. During the experiment, the continuous phase β-Lg in the control sample did not reveal any additional protection against the formation of lipid hydroperoxides at any storage time. BCA alone at concentration level of 20 μM were able to inhibit the formation of lipid hydroperoxides by 31% during the later stages of oxidation with 50 μM BCA being more effective with 50% inhibition level ($p < 0.05$) (Fig. 1). However, β-Lg did not improve the ability of BCA to inhibit lipid hydroperoxides. The lag phase of lipid hydroperoxide formation was also not changed by RE (Fig. 2). During the later stages of oxidation, the antioxidant activities of RE alone at 20 and 50 μM were 34 and 49%, respectively, toward lipid hydroperoxides when compared to the control sample. However, no differences in activity were observed between the 20 and 50 μM RE treatments when β-Lg was incorporated into the emulsions. There is contradictory data available on how proteins affect the reactivity of polyphenols. Protein–polyphenol interactions have been shown to be dependent on pH, temperature, and protein and polyphenol concentrations [38]. A study by Arts et al. [39] revealed that polyphenols may not necessarily reach their optimum antioxidant activities in a protein matrix. They suggested that protein–polyphenol interactions may reduce the antioxidant activity of the phenolic compounds. Another study by Riedl and Hagerman [40] showed that polyphenols can be potent antioxidants even when bound to proteins. It has been proposed that the binding between phenolics and protein matrix might account for the enhancement of antioxidant capacity, since protein–phenolic interaction is able to stabilize the protein and its antioxidant capacity is increased during heating [41]. It has been suggested that tannins could bind by coating the protein surface [42]. In the presence of an excess of polyphenol relative to the protein, all of the protein interactions sites would be occupied and the free polyphenol would have a small possibility of finding a free interaction site on a protein molecule [43]. Isolated mixtures of chestnut and myrabolan ellagitannins exhibited three to fourfold greater binding affinities for interactions with gelatine than for BSA, whereas tara and sumac gallotannins bound with equal strength to both gelatine and BSA. The differences in the binding constants were dependent on the structural flexibility of the tannin molecule. As the loss of conformational freedom of ellagitannins appears not to interfere with binding a flexible protein such as gelatine, however, the flexibility of the protein plays an important role in the protein–phenolic binding [42]. However, further research is needed to elucidate the exact dose–response relationship of protein–polyphenol interactions.

Hexanal formation in different Brij 35-stabilized corn oil-in water emulsions (pH) at 55 °C during 24 days are shown in Figs. 3 and 4. In the control emulsion sample, a lag phase of 6–8 days was observed followed by a rapid rise in hexanal formation. The rapid rise in secondary lipid oxidation products is commonly seen after the radical chain propagation of the primary lipid oxidation products. This is because lipid hydroperoxides are an unstable intermediate product of lipid oxidation that can be decomposed by factors

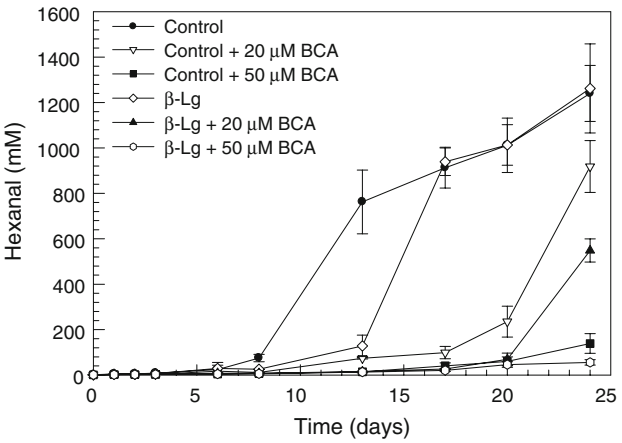

Fig. 3 Formation of hexanal in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with and without continuous phase *β-lactoglobulin* (*β-Lg*, 0.5 mg/g oil) with added black currant anthocyanins (*BCA*) during oxidation at 55 °C in the dark (mM, means ± SD, $n = 3$)

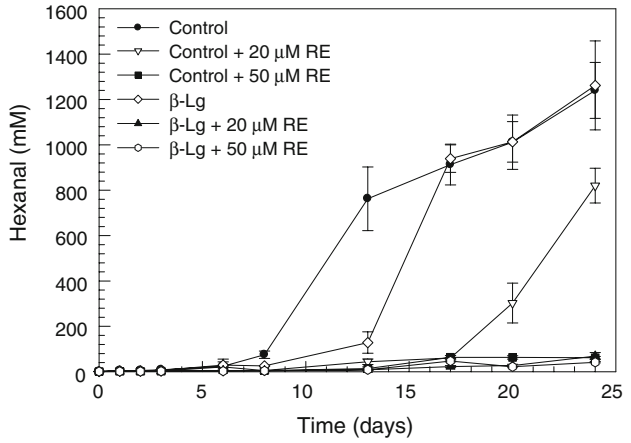

Fig. 4 Formation of hexanal in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with and without continuous phase *β*-lactoglobulin (*β-Lg*, 0.5 mg/g oil) with added raspberry ellagitannins (*RE*) during oxidation at 55 °C in the dark (mM, means ± SD, $n = 3$)

such as transition metals, light and high temperatures [2]. Decomposition of lipid hydroperoxides results in the formation of alkoxyl radicals which can then cause fatty acid *β*-scission leading to the formation of the small, volatile oxidation products such as aldehydes, acids, alcohols, and short-chain hydrocarbons that cause rancidity in processed foods, especially in oil-in-water emulsions. According to the experimental data, continuous phase *β*-Lg prolonged the lag phase of hexanal formation from 6 days of the control to 13 days. Corn oil-in-water emulsion in the presence of berry phenolics and berry phenolics in combination with the continuous phase *β*-Lg extended the lag phase significantly compared to the control emulsion samples (Figs. 3, 4). The antioxidant activity toward hexanal formation was 26 and 89% in the presence of BCA alone (Fig. 3) and 34 and 95% in the presence of RE alone (Fig. 4) at concentration levels of 20 and 50 μM, respectively. However, the combination of *β*-Lg and BCA was more effective ($p < 0.05$) inhibiting hexanal formation than either component alone. In the presence of aqueous phase *β*-Lg, BCA at 20 and 50 μM exhibited antioxidant activities of 56 and 96%, respectively. The results also show that combination of *β*-Lg and RE at 20 μM was more efficient at inhibiting hexanal formation (by 94%) than either component alone ($p < 0.05$) (Fig. 4). RE at 50 μM alone and in combination with *β*-Lg were very effective at inhibiting the formation of hexanal by 95 and 97%, respectively. However, at this concentration level (50 μM of RE) no statistical differences in the antioxidant activities were found between samples with or without continuous phase *β*-Lg.

The results show that large differences were observed for the ability of continuous phase *β*-Lg and the berry phenolics to inhibit lipid hydroperoxides and hexanal formation in the oil-in-water emulsions. Although the berry phenolics showed a moderate antioxidant activity toward inhibiting lipid hydroperoxides, the inability of *β*-Lg to decrease lipid hydroperoxide formation suggests that these additives were increasing lipid hydroxide decomposition possibly by increasing the solubility and/or reactivity of iron (via reduction) [2]. However, berry phenolics alone and in combination with *β*-Lg were able to inhibit hexanal formation suggesting that they were able to scavenge alkoxyl radicals to decrease fatty acid scission. This is important because 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 off-flavors [18]. Our results also indicate that the combination of *β*-Lg and berry phenolics are more effective at inhibiting fatty acid scission than individual phenolic antioxidants. The results also suggest that the antioxidant activity of berry phenolics at the 20 μM level in combination with continuous phase *β*-Lg may be due to synergism. However, there were no synergistic effects of antioxidant activity at the 50 μM concentration level of berry phenolics in combination with continuous phase *β*-Lg. This data is in accordance with earlier studies showing proteins such as BSA enhancing the oxidative stability of oil-in-water emulsions in the presence of green tea catechins even though BSA itself did not act as antioxidant [15, 29]. This synergistic effect was suggested as being due to the changed structure of BSA, with a loss of tryptophan groups, and the formation of BSA-antioxidant adducts, which concentrate at the oil–water interface due to the surface-active properties of the protein [15, 29]. It is commonly accepted that the overall antioxidant mechanism of flavonoids is recognized as a combination of a direct reaction with free radicals and chelation of metal ions [11, 12]. In addition, it has been postulated that phenolic compounds can retard oxidation reactions by binding to proteins and by forming complexes between protein molecules [40, 44]. Larger molecular weight phenolics such as ellagitannins have the ability to bind to the protein more efficiently due to the proximity of many aromatic rings and hydroxyl groups, and increase association of antioxidants at the surface of the emulsion droplets, and thus inhibit oxidation [29]. In addition, it has been shown that phenolic compounds can bind to bovine and reindeer *β*-Lg [45]. Anthocyanins, however, have been suggested to bind only to specific glutathione *S*-transferase—proteins in grape berries (*Vitis vinifera* L.) [46] and petunia (*Petunia hybrida*) [47]—in the transport of anthocyanins from the cytosol to the plant vacuole.

Amino Acid Oxidation

Berry phenolics were found to interfere with the measurements of both tryptophan and cysteine fluorescence.

RE caused a decrease in the tryptophan fluorescence with a blue-shift from 331 to 315 nm, which was the emission maximum of RE (Fig. 5a). There was no difference in the fluorescence intensities between RE by themselves or RE with the protein. The decrease in fluorescence indicates that the RE bound to the protein. In contrast, BCA caused a red-shift in the β-Lg (Fig. 5b) from 331 to 350 nm, which indicated the maximum emission wavelength of BCA. Some studies have reported that berry phenolics did not cause fluorescence quenching of tryptophan [13, 14]. This may be due to much higher protein concentrations used compared to the phenolics. Since berry phenolics interfered with the fluorescence measurements, the protein was separated from the phenolic compounds by chromatography matrix retaining smaller than 6,000 Da molecules, and the absence of phenolic compounds was confirmed by fluorescence spectroscopy. After separation with the desalting column, the total protein concentrations of the samples were determined. Due to the differences in the initial protein concentrations between the samples, the results were normalized accordingly, and expressed relative to day 0. The proportion of the protein that was modified during oxidation remained unknown.

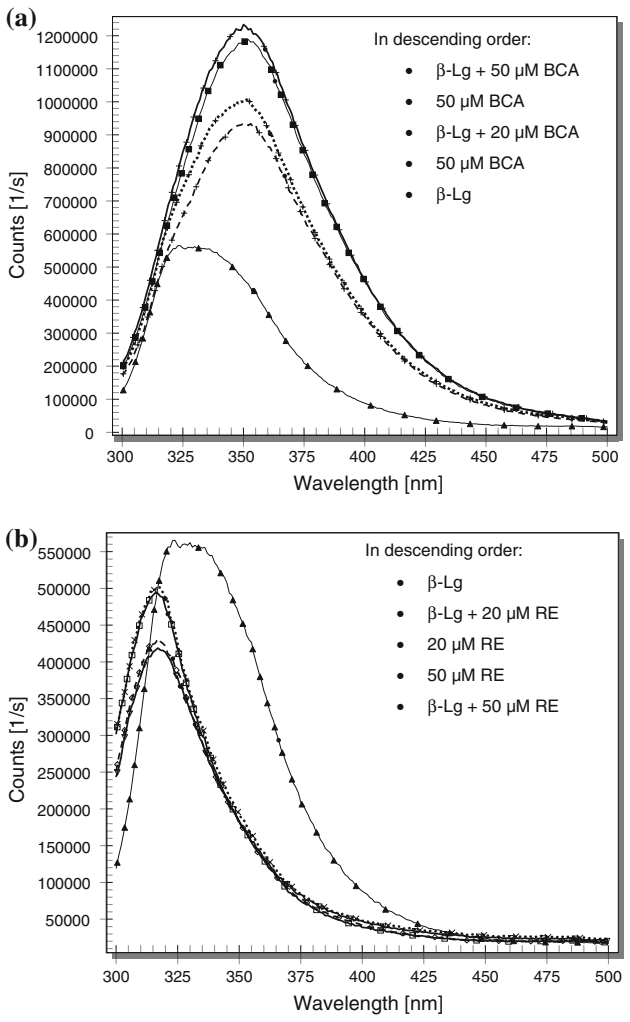

Fig. 5 Emission spectra of β-lactoglobulin (β-*Lg*) (25 μg/mL) at $\lambda_{Ex} = 283$ nm (pH 7.0) in the presence of different concentrations of **a** raspberry ellagitannins (*RE*) and **b** black currant anthocyanins (*BCA*)

The amino acid oxidation of tryptophan and cysteine of continuous phase β-Lg was investigated in Brij 35-stabilized corn oil-in-water emulsions (pH 7) with or without berry phenolics at 55 °C during 24 days (Figs. 6, 7). Cysteine, tryptophan, histidine, lysine, tyrosine, and arginine are the primary amino acids in proteins that react with reactive oxygen species due to their readily abstractable hydrogens and hydrogen-bonding properties [5, 48]. The susceptibility of these amino acid residues in proteins to oxidation is dependent on their location in the protein which dictates whether they are exposed to the aqueous and lipid media [26–28]. β-Lg consists of 162 amino acid residues (18.3 kDa) and contains two disulfide bonds (Cys66-Cys160 and Cys106-Cys119), a free thiol (Cys121), and two tryptophan residues (Trp19 and Trp61). The reactivity of tryptophan residues in β-Lg is usually limited to 50% [49] because Trp19 is completely buried and Trp61 is exposed. The limited fluorescence of Trp19 has been explained by the nearby Arg124 residue quenching its signal [49]. As shown in Fig. 6, the tryptophan fluorescence decreased by 44% after 2 days of oxidation ($p < 0.05$) in the emulsion sample containing only β-Lg. The reduction in tryptophan fluorescence by $\sim$40% in β-Lg during oxidation is consistent with previous studies [27], and is most likely due to oxidation of Trp61. After day 6, a further decrease ($p < 0.05$) in tryptophan fluorescence occurred, and after 13 days of oxidation 82% of

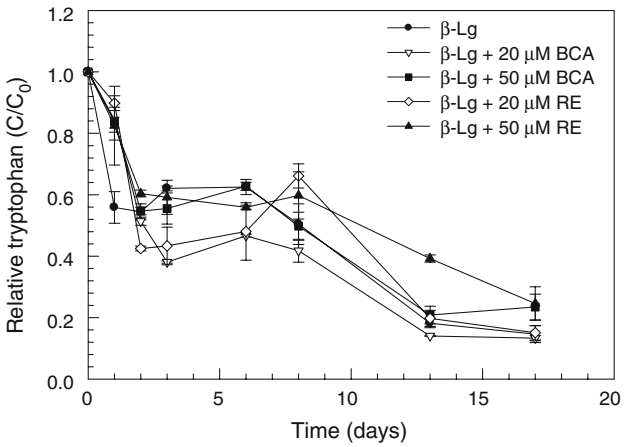

Fig. 6 Loss of tryptophan in continuous phase β-lactoglobulin (β-*Lg*) in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with added black currant anthocyanins (*BCA*) and raspberry ellagitannins (*RE*) at concentration levels of 20 and 50 μM during oxidation at 55 °C in the dark. Unoxidized tryptophan residue concentrations (C) are expressed relative to their day 0 concentrations (C_0) (means ± SD, $n = 3$)

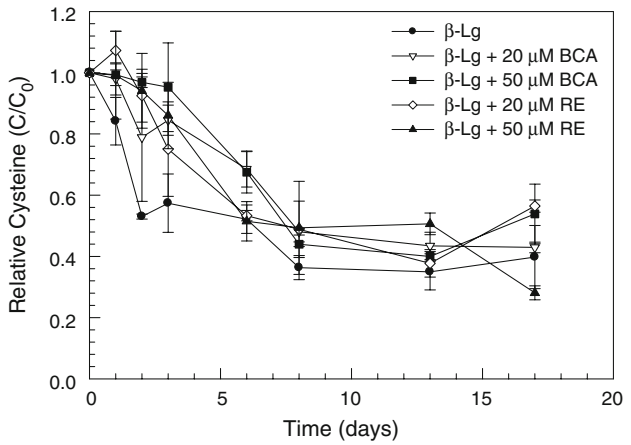

Fig. 7 Loss of cysteine in continuous phase *β*-lactoglobulin (*β-Lg*) in Brij 35-stabilized 5% corn oil-in-water emulsions (pH 7.0) with added black currant anthocyanins (*BCA*) and raspberry ellagitannins (*RE*) at concentration levels of 20 and 50 μM during oxidation at 55 °C in the dark. Unoxidized cysteine residue concentrations (*C*) are expressed relative to their day 0 concentrations (C_0) (means ± SD, $n = 3$)

tryptophan was oxidized (Fig. 6). In native *β*-Lg, free Cys121 is buried within the hydrophobic core, and therefore has low reactivity [49]. However, a heat treatment of *β*-Lg has been shown to increase the solvent accessibility of sulfhydryl groups [28]. A rapid loss of cysteine residues by 47 and 64% was observed after 2 and 8 days of oxidation ($p < 0.05$), respectively (Fig. 7). After that, further oxidation of cysteine residues did not occur.

Both tryptophan and cysteine in the continuous phase *β*-Lg were substantially oxidized prior to the decomposition of fatty acids to form hexanal. This suggests that these amino acids are able to inhibit fatty acid scission. These results are consistent with a study by Elias et al. [27] who observed that cysteine and tryptophan residues were oxidized before lipid oxidation was detected. In addition, they showed that cysteine residues oxidized more rapidly than Trp. In contrast, this study revealed that tryptophan was oxidized more rapidly than cysteine. This may be due to differences in oxidation temperature (37 vs. 55 °C), which could impact protein conformation.

The influence of berry phenolics on the oxidation of continuous phase *β*-Lg in the emulsion was investigated subsequently. BCA at 20 and 50 μM inhibited the oxidation of tryptophan residues only until the second day of oxidation ($p < 0.05$) (Fig. 6). After this, loss of tryptophan fluorescence was more pronounced with 20 μM BCA than that of *β*-Lg. *β*-Lg with 50 μM BCA showed a similar pattern in tryptophan fluorescence as without the phenolics. RE at 20 μM were able to inhibit the oxidation of tryptophan residues only until the second day of oxidation whereas RE at 50 μM inhibited tryptophan oxidation until the third day ($p < 0.05$). RE showed an increase in the fluorescence intensity at day 8, later than for the *β*-Lg itself, indicating a possible unfolding of the protein structure. Thus, the exposure of the buried amino acids residues enabled them to act as antioxidants, and retard the lipid oxidation reactions.

The effect of berry phenolics was more pronounced in inhibiting the oxidation of cysteine residues (Fig. 7) compared to the control. BCA (at 20 and 50 μM) were able to inhibit cysteine oxidation for 6 days ($p < 0.05$), and after 8 days of oxidation there were no differences in cysteine oxidation between the protein sample and the protein with the added BCA. RE at 50 μM were able to inhibit cysteine oxidation for 3 days ($p < 0.05$), and after 6 days of oxidation cysteine was fully oxidized. RE at 20 μM inhibited the oxidation of cysteine only 2 days ($p < 0.05$). Consequently, these results corroborate that the radical transfer to proteins is high in the beginning of oxidation when lipid oxidation appears to be low.

Based on the results in this study, berry phenolics such as BCA and RE showed potent antioxidant activities toward lipid and protein oxidation reactions in corn oil-in-water emulsions. The antioxidant effect of berry phenolics in combination with the aqueous phase *β*-Lg toward hexanal formation was more pronounced than without *β*-Lg. The amino acid oxidation data showed that both tryptophan and cysteine in the continuous phase *β*-Lg were substantially oxidized prior lipid oxidation. In addition, both berry phenolics were able to retard the oxidation of the amino acid residues in *β*-Lg. Our results contribute to elucidating the effects of natural phenolic compounds as potential antioxidants in order to control and prevent protein and lipid oxidation reactions in oil-in-water emulsions. Understanding the relationship between phenolic compounds and proteins as well as lipids could lead to the development of new and effective antioxidant strategies that could be used in food, cosmetic and pharmaceutical applications. More research, however, is needed to elucidate the exact interactions between the individual ingredients present in emulsions systems.

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