JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Anthocyanin Antioxidant Activity and Partition Behavior in Whey Protein Emulsion

KAARINA VILJANEN,*,† PETRI KYLLI,† EVA-MARIA HUBBERMANN,‡ KARIN SCHWARZ,‡ AND MARINA HEINONEN†

Department of Applied Chemistry and Microbiology, Division of Food Chemistry, University of Helsinki, P.O. Box 27, FIN-00014 Helsinki, Finland, and Institute of Human Nutrition and Food Science, University of Kiel, Heinrich-Hecht-Platz 10, 24098 Kiel, Germany

The antioxidant activities of anthocyanins and anthocyanin fractions isolated from blackcurrants, raspberries, and lingonberries were investigated in whey protein-stabilized emulsion. The extent of protein oxidation was measured by determining the loss of tryptophan fluorescence and formation of protein carbonyl compounds and that of lipid oxidation by conjugated diene hydroperoxides and hexanal analyses. The antioxidant activity of berry anthocyanins increased with an increase in concentration. Blackcurrant anthocyanins were the most potent antioxidants toward both protein and lipid oxidation at all concentrations due to the beneficial combination of delphinidin and cyanidin glycosides. Most berry anthocyanins (69.4−72.8%) partitioned into the aqueous phase of the emulsion, thus being located favorably for antioxidant action toward protein oxidation. The presence of the lipid decreased the share of anthocyanin in the aqueous phase. Thus, the structure of food affects the antioxidant activity by influencing the partitioning of the antioxidant.

KEYWORDS: Protein oxidation; anthocyanins; antioxidants; partition; emulsion; berries

INTRODUCTION

Milk proteins are used in various food products because of their excellent emulsifying and foaming properties. During homogenization, proteins are able to absorb to the surface of oil droplets. They lower interfacial tension and inhibit droplets' coalescence by forming protective membranes around the oil droplets. The stability of emulsion depends on noncovalent interactions, hydrophobic and electrostatic interactions, and hydrogen bonding between adsorbed proteins (*1*). In addition, both intra- and intermolecular disulfide bonds play an important role in protein-stabilized emulsions (*2*).

In food products, lipid oxidation can cause protein oxidation due to close interactions between lipids and proteins. During oxidation, whey proteins can cross-link and therefore affect the texture of food, i.e., change the viscosity of a solution (*3*, *4*). Oxidation reactions affect the quality of food, but they also have an impact on the charge and conformation of protein three-dimensional structure (exposure of hydrophobic groups, changes in secondary structure, and disulfide groups) and protein functionality such as changes in food texture, decreases in protein solubility (due to aggregation or complex formation), color changes (browning reactions), loss of enzyme activity, and changes in nutritive value (loss of essential amino acids) (*5*, *6*).

In β-casein- and β-lactoglobulin-stabilized oil-in-water emulsions, more than 99% of the protein modification is supposed to occur via Michael addition reactions (*7*, *8*). The carbonyl group of the aldehydes may subsequently participate in the formation of intra- and possibly intermolecular cross-links with amino acid residues via covalent bonding (via formation of Schiff bases). The reactions between aldehydes and amino groups can occur in emulsions at the oil−water interface with the initial sites of modification being located in the hydrophobic regions of the protein molecules. As the emulsion ages, the extent of oxidation and hence of modification increases and the protein becomes more firmly anchored to the oil phase via carbonyl groups. These modified proteins will have functional properties different from those of their unmodified molecules; their emulsifying, gelling, and water binding properties may be affected (*8*). The physical stability of an emulsion depends also on the concentration of protein in solution, pH, and temperature (*9*−*11*).

Casein has been shown to stabilize emulsion more than whey proteins, and both proteins have shown antioxidant activity as measured by inhibition of formation of lipid oxidation products (*12*−*15*). Casein, lactalbumin, and BSA (bovine serum albumin) have also been shown to inhibit liposome oxidation (*16*). The antioxidant activity of proteins is directly linked to the ability of its amino acid residues to react with lipid free radicals and hydroperoxides. The difference in the oxidative stabilities of different emulsions stabilized by proteins is due to differences in amino acid composition among proteins as well. Some amino

* To whom correspondence should be addressed. Telephone: +358 9 191 59794. Fax: +358 9 191 58475. E-mail: kaarina.viljanen@helsinki.fi.
† University of Helsinki.
‡ University of Kiel.

10.1021/jf047975d CCC: $30.25
Published on Web 02/24/2005

acids bind more strongly to oil droplets, or chelate more transition metals, and therefore inhibit more lipid oxidation (*13*, *14*, *17*).

Oxidation of proteins by lipid oxidation products can furthermore lead to oxidation of amino acid residue side chains, cleavage of peptide bonds, and formation of covalent protein−protein cross-linked derivatives (*18*). Oxidative cleavage of the peptide bond in the main chain leads to formation of peptide fragments and the oxidation of the side chains of lysine, proline, tryptophan, arginine, and threonine, yielding protein−carbonyl compounds.

In foods, phenolic compounds also can bind to the proteins by hydrogen bonding between the carbonyl group of the protein−peptide bond and the phenolic hydroxyl group in tannin (*19*−*21*). The formed complexes are stabilized by proline residues and hydrophobic interactions. However, tryptophan, lysine, and methionine can take part in the interaction reactions which leads to the limited availability of the essential amino acids. The reaction between phenolic compounds and protein can also lead to a loss of protein solubility and changes in protein hydrophobicity. In addition, the interaction reaction can affect the protein isoelectric point, which is shifted to lower pH values due to the introduction of carboxylic groups following the covalent attachment of the phenolic acids and by the parallel blocking of lysine residues in protein (*21*).

The aim of this study was to investigate berry anthocyanin antioxidant activity and partition behavior between oil and aqueous phases, and affinity for proteins in whey protein emulsion. In earlier studies, anthocyanins and different berries have been shown to inhibit both lipid and protein oxidation in a liposome model system (*16*, *22*, *23*). In emulsions, the proportions of antioxidants residing in different phases depend on the relative polarity of the antioxidants and the lipid substrates, surfactants, pH, and temperature as well as the composition of the phases (*24*−*26*). The antioxidant activity in emulsions was investigated by assessing both protein and lipid oxidation.

EXPERIMENTAL PROCEDURES

Materials. Cyanidin (Cya), delphinidin (Del), pelargonidin (Pel), and their glucosides were obtained from Extrasynthèse (Genay, France), except for delphinidin 3-glucoside and delphinidin 3-rutinoside which were from Polyphenols (Sandnes, Norway). Whey protein product (DSE 5323), containing 38% β-lactoglobulin, 9% α-lactalbumin, and 12% other whey proteins, was obtained from a New Zealand milk product. Copper(II) acetate and sulfosalisylic acid were from Merck (Darmstadt, Germany). AAS-grade ethanol was from Primalco (Rajamäki, Finland), and all other HPLC-grade solvents were from Rathburn (Walkerburn, U.K.). The rapeseed oil (Kultasula, Mildola, Finland) was purified prior to being used as described by Lampi et al. (*27*). The citrate buffer was made of citric acid (Pharmia Ltd., Helsinki, Finland) and sodium hydroxide (Dilut-it, J. T. Baker, Deventer, The Netherlands) adjusted to pH 5.4. The water was purified by a Milli Q system (Millipore, Bedford, MA). Amberlite XAD-7 nonionic polymeric adsorbent was purchased from Sigma Chemical Co. (St. Louis, MO). All berries, blackcurrant (*Ribes nigrum* var. Öjebyn), raspberry (*Rubus idaeus*), and lingonberry (*Vaccinium vitis-idaea*), were purchased from a local market. The leaves and branches were picked from berry samples, and they were packed immediately into a vacuum and stored at −18 °C until they were used.

Extraction and Isolation of Berry Anthocyanins. Anthocyanin fractions from blackcurrants, raspberries, and lingonberries were isolated as described by Kähkönen et al. (*28*). Extraction of phenolic compounds was carried out by homogenization (Ultra-Turrax T25 mixer, Janke & Kunkel) for 1 min with 2 g of berries with 20 mL of solvent [49.5:0.5:50 (v/v/v) CH_3CN/TFA/H_2O] in a centrifuge tube. The homogenates were centrifuged (4000 rpm for 15 min), and the clear supernatant was collected. The procedure was repeated twice with another 20 mL of solvent (berry phenolic extracts). The supernatants were combined and dried. The solid residues were dissolved in 0.5% TFA (pH 1.5).

Berry anthocyanins were isolated by the method described by Kähkönen et al. (*28*) by using Amberlite XAD-7 column chromatography (diameter of 40 mm, length of 300 mm). First, free sugars, organic acids, and phenolic acids were eluted from berry phenolic extracts with 6% CH_3CN [6.0:0.5:93.5 (v/v/v) CH_3CN/TFA/H_2O]. Then the anthocyanin-containing fractions were eluted with 50% CH_3CN [50:0.5:49.5 (v/v/v) CH_3CN/TFA/H_2O], and finally, the column was washed with CH_3CN [99.5:0.5 (v/v) CH_3CN/TFA] to elute the remaining phenolics. Anthocyanin fractions were further purified by using preparative HPLC, and the anthocyanin composition was analyzed with HPLC as described by Kähkönen et al. (*28*).

Preparation of Emulsions. The 10% (w/w) oil-in-water emulsions were prepared by sonicating 1 g of purified rapeseed oil, 0.20 g of whey protein concentrate, and 9 mL of citrate buffer (pH 5.4) for 3 min with a U 50 Control Ikasonic Sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Germany) in an ice bath. Purified rapeseed oil was checked to be free of tocopherols before use with a HPLC method described by Haila and Heinonen (*29*). Anthocyanins (at levels of 50 and 200 μM corresponding to approximately 250 and 1000 μg/g, respectively) and anthocyanin fractions isolated from blackcurrants, raspberries, and lingonberries (at levels of 50, 100, and 500 μg/g) were dissolved in ethanol and pipetted into glass vials (20 mL). The ethanolic solution was evaporated with nitrogen, and the rapeseed oil/protein emulsion (10 mL) and 3 μM copper acetate solutions were added to the vials. The emulsion in the sealed vials was oxidized in the dark at 37 °C for 4 days.

Lipid Oxidation. Lipid oxidation was evaluated by formation of conjugated diene hydroperoxides and hexanal. Samples (25 μL) were dissolved in 5 mL of isooctane, and conjugated diene hydroperoxides were analyzed spectrophotometrically at 234 nm (Lambda Bio UV/VIS spectrophotometer, Perkin-Elmer). The amount of hexanal (250 μL samples) was measured using a static headspace gas chromatograph (Autosystem XL gas chromatograph equipped with an HS40XL headspace sampler, Perkin-Elmer, Shelton, CT; column NB-54, Nordion) according to the method of Frankel et al. (*30*). The percent inhibition against lipid oxidation was calculated on day 4 using the same formula as for inhibition of protein carbonyls: $[(C_t - S_t)/C_t] \times 100$, where C_t is the amount of conjugated diene hydroperoxides or hexanal in control sample at time t and S_t is the amount of conjugated diene hydroperoxides or hexanal in the antioxidant sample at time t. The results are represented as the mean values of triplicate analysis.

Protein Oxidation. Protein oxidation was assessed by fluorescence spectroscopy by following both the formation of protein carbonyls and the loss of natural tryptophan fluorescence (*16*, *22*, *31*). Samples (100 μL) were dissolved in 1 mL of citrate buffer. Emission spectra of tryptophan were recorded from 300 to 400 nm with the excitation wavelength set at 283 nm (F-4010 Hitachi fluorescence spectrophotometer). In addition, emission spectra of latter products of oxidation (protein carbonyls) were recorded from 400 to 500 nm with the excitation wavelength set at 350 nm. The percent inhibition against loss of tryptophan fluorescence was calculated at day 4 as $[(C_0 - C_t) - (S_0 - S_t)]/(C_0 - C_t) \times 100$, where C_0 is the initial fluorescence of the control sample, C_t is the fluorescence of the control sample at time t, S_0 is the initial fluorescence of the antioxidant sample, and S_t is the fluorescence of the antioxidant sample at time t. The percent inhibition of protein carbonyls was calculated on day 4 as $[(C_t - S_t)/C_t] \times 100$, where C_t is the fluorescence of protein carbonyls in the control sample at time t and S_t is the fluorescence of protein carbonyls in the antioxidant sample at time t. All results are represented as the mean values of triplicate analysis.

Fourth derivatives of tryptophan spectra were calculated according to the Stavitzky−Golay modified procedure to obtain the result of tryptophan oxidation in different environments (hydrophobic and hydrophilic). The ratio of intensities at 310 and 326 nm of the fourth-derivative spectra (d^4_{310}/d^4_{326}) was calculated by applying the peak−peak method (*32*).

Table 1. Anthocyanin Composition of Raspberry, Blackcurrant, and Lingonberry Anthocyanin Fractions (Expressed as a Percentage of Total Anthocyanins Measured Using HPLC)

anthocyanin[a]	raspberry	blackcurrant	lingonberry
Pel-3-glu	0.9		
Pel-3-rut	0.7		
Pel-3-soph	2.5		
Cya-3-glu	15.6	7.0	78.6
Cya-3-rut	5.3	37.8	
Cya-3-soph	59.4		
Cya-3-gal			3.2
Cya-3-ara			18.2
Cya-3-glurut	15.6		
Del-3-glu		15.8	
Del-3-rut		38.9	
Peo-3-rut		0.5	

[a] Abbreviations: Pel, pelargonidin; Cya, cyanidin; Del, delphinidin; Peo, peonidin; glu, glucoside; rut, rutinoside; soph, sophoriside; gal, galactoside; ara, arabinoside; glurut, glucosylrutinoside.

Partition of Anthocyanins in Emulsion. Three model systems were used for assessing partitioning of blackcurrant, raspberry, and lingonberry anthocyanins between different phases: (a) 10% (w/w) rapeseed oil/citrate buffer (pH 5.4) mixtures, (b) 2% (w/w) whey protein solutions in citrate buffer (pH 5.4), and (c) 10% (w/w) oil-in-water emulsions with 2% (w/w) whey protein concentrate as described previously. The partition of the anthocyanins at the concentration of 500 μg/g in three model systems was assessed as described by Schwarz et al. (*24*) by using ultrafiltration centrifuge tubes with a molecular weight cutoff of 3000 (Centricon YM-3, Millipore). The clear filtered sample (50 μL) was diluted to 1 M sulfosalisylic acid (108 μL) before HPLC analysis. The partition between different phases were calculated as $[(E - S \times C)/E] \times 100$, where E is the amount of anthocyanins in the emulsion sample, S is the amount of anthocyanin in the permeate (aqueous phase) after ultrafiltration, and C is the amount of oil and/or protein in the sample. The results are given as the mean values of triplicate analysis.

HPLC Analysis of Anthocyanins. The anthocyanins before and after partitioning were analyzed by using a method described by Kähkönen et al. (*28*). Emulsion samples (2 mL) were first centrifuged with 400 μL of 3 M sulfosalisylic acid for 15 min at 3500 rpm prior to anthocyanin analysis of the clear supernatant by HPLC.

Statistical Analysis. Statistical differences among antioxidant activities were tested by multivariance analysis using Statgraphics Plus (STCC Inc., Rockville, MD). The significance level was $p < 0.05$.

RESULTS

Anthocyanin Composition of Different Berries. The total amount of anthocyanins and the anthocyanin profile of raspberries, blackcurrants, and lingonberries are shown in **Table 1**. Raspberries and blackcurrants exhibited a more complex anthocyanin profile than lingonberries. Lingonberries contained only different cyanidin glycosides, whereas raspberries also contained pelargonidin glycosides and blackcurrant delphinidin glycosides.

Effect of Anthocyanins on Lipid Oxidation. Anthocyanins inhibited lipid oxidation at most with 76% inhibition of hexanal formation (pelargonidin 3-glucoside) (**Table 2**). In general, the antioxidant effect of anthocyanins in emulsions was only moderate. Stronger inhibition was seen toward the formation of hexanal compared to conjugated diene hydroperoxide formation. At concentration of 200 μM, cyanidin 3-rutinoside and pelargonidin 3-glucoside were the most potent antioxidants toward formation of hexanal. At both tested concentrations (50 and 200 μM), the rutinoside forms of anthocyanins inhibited more formation of conjugated diene hydroperoxides than the glucoside forms, although the differences were not always significant.

Table 2. Inhibition of Conjugated Diene Hydroperoxides and Hexanal Formation by Anthocyanins Tested in a Whey Protein Emulsion System after Oxidation for 4 Days (Percent Inhibition, Mean ± Standard Deviation)[a]

	conjugated dienes		hexanal	
compound[b]	50 μM	200 μM	50 μM	200 μM
Cya	20.1 ± 0.5 bc	41.8 ± 0.5 a	39.3 ± 0.7 c	44.1 ± 1.0 cd
Cya-3-glu	21.2 ± 3.4 abc	–2.1 ± 0.2 d	72.1 ± 0.9 a	68.2 ± 0.2 ab
Cya-3-rut	30.4 ± 7.8 abc	31.1 ± 0.9 ab	58.1 ± 0.9 b	75.2 ± 3.9 a
Del	15.0 ± 3.7 c	25.3 ± 1.7 bc	65.1 ± 7.9 ab	29.8 ± 5.1 e
Del-3-glu	19.5 ± 3.3 abc	21.0 ± 9.7 bc	54.7 ± 7.6 b	–0.3 ± 5.4 f
Del-3-rut	30.8 ± 3.7 ab	29.5 ± 1.8 ab	65.5 ± 3.0 ab	38.7 ± 0.3 de
Pel	33.2 ± 4.0 ab	11.5 ± 11.1 d	67.6 ± 5.9 ab	56.0 ± 1.4 bc
Pel-3-glu	36.5 ± 1.2 a	12.3 ± 3.0 cd	67.9 ± 3.2 ab	76.1 ± 1.8 a

[a] Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$). Negative values indicate prooxidant activity. [b] Abbreviations: Cya, cyanidin; Del, delphinidin; Pel, pelargonidin; glu, glucoside; rut, rutinoside.

Table 3. Inhibition of Tryptophan Loss and Carbonyl Formation (after Oxidation for 4 Days) by Anthocyanins Tested in a Whey Protein Emulsion System (Percent Inhibition, Mean ± Standard Deviation)[a]

	tryptophan loss		carbonyl gain	
compound[b]	50 μM	200 μM	50 μM	200 μM
Cya	3.2 ± 0.3 bc	8.7 ± 2.2 b	28.4 ± 0.4 e	35.4 ± 2.5 d
Cya-3-glu	7.5 ± 2.2 ab	2.2 ± 1.8 bc	59.7 ± 1.5 ab	90.0 ± 1.0 a
Cya-3-rut	13.3 ± 2.6 a	–7.4 ± 0.5 d	56.2 ± 3.2 b	55.1 ± 4.2 bc
Del	0.7 ± 1.6 bc	14.7 ± 3.1 a	50.2 ± 2.6 bc	50.9 ± 6.4 bc
Del-3-glu	–1.2 ± 1.3 cd	9.4 ± 5.5 b	43.0 ± 1.1 cd	94.0 ± 0.2 a
Del-3-rut	1.7 ± 1.3 bc	7.5 ± 0.4 bc	68.8 ± 1.4 a	69.1 ± 6.5 b
Pel	–7.0 ± 1.8 d	13.3 ± 1.0 a	48.8 ± 1.1 c	67.4 ± 1.6 b
Pel-3-glu	6.5 ± 0.2 ab	4.4 ± 1.5 bc	50.5 ± 2.1 bc	59.3 ± 0.5 bc

[a] Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$). Negative values indicate prooxidant activity. [b] Abbreviations: Cya, cyanidin; Del, delphinidin; Pel, pelargonidin; glu, glucoside; rut, rutinoside.

Effect of Anthocyanins on Protein Oxidation. Anthocyanins acted as weak antioxidants or prooxidants toward the loss of tryptophan fluorescence (**Table 3**). The highest level of inhibition (14.7%) was reached by delphinidin aglycon at 200 μM. All anthocyanins were more potent antioxidants toward formation of protein carbonyl compounds than toward loss of tryptophan fluorescence. Delphinidins were the best antioxidants at concentrations of 50 μM (rutinoside) and 200 μM (glucoside) toward formation of carbonyl compounds. Glycosylation increased the antioxidant activity compared to respective aglycon forms except for delphinidin 3-glucoside at 50 μM and perlargonidin 3-glucoside at 200 μM. At both concentrations, glucoside forms were more potent than rutinoside forms except for delphinidins at 50 μM.

Effect of Berry Anthocyanin Fractions on Lipid and Protein Oxidation. Berry anthocyanins were potent antioxidants toward both protein and lipid oxidation at high concentration of 500 μg/g (**Table 4**). The overall antioxidant activity of berry anthocyanins decreased in the following order: blackcurrant > lingonberry > raspberry. Blackcurrant anthocyanins were the only anthocyanins that did not exhibit a prooxidant effect at a concentration of 100 μg/g, while at a concentration of 50 μg/g, all berry anthocyanin isolates promoted emulsion oxidation. Raspberry and lingonberry anthocyanin isolates promoted whey protein emulsion oxidation at concentrations of 50 and 100 μg/g; however, they at least to a moderate degree prevented both protein and lipid oxidation at the highest tested concentration

Table 4. Inhibition of Protein and Lipid Oxidation by Anthocyanin Fractions Isolated from Berries Tested in a Whey Protein Emulsion after Oxidation for 4 Days (Percent Inhibition, Mean ± Standard Deviation)[a]

	μg/g	tryptophan loss	carbonyl gain	hydroperoxides	hexanal
blackcurrant	50	9.9 ± 0.7 b	45.7 ± 1.5 d	−2.1 ± 2.3 c	22.8 ± 0.9 e
	100	5.7 ± 0.8 c	65.3 ± 0.4 c	40.7 ± 6.8 b	67.5 ± 1.9 b
	500	12.2 ± 0.5 bc	82.1 ± 0.4 a	52.4 ± 1.1 a	93.5 ± 0.1 a
raspberry	50	−3.1 ± 0.2 d	15.5 ± 0.6 e	−12.9 ± 2.9 cd	−18.1 ± 2.3 f
	100	−1.9 ± 0.1 d	14.4 ± 0.1 e	−5.4 ± 1.7 c	−37.5 ± 1.2 g
	500	9.8 ± 0.1 b	71.4 ± 0.2 b	50.9 ± 0.9 ab	40.0 ± 1.2 d
lingonberry	50	−2.3 ± 0.2 d	7.5 ± 2.2 f	−17.8 ± 4.7 d	−50.4 ± 0.9 h
	100	−0.9 ± 0.1 b	13.5 ± 0.4 e	−2.5 ± 4.0 c	−38.6 ± 0.8 g
	500	6.8 ± 0.5 bc	82.5 ± 0.5 a	45.3 ± 0.4 ab	49.3 ± 0.6 c
α-tocopherol	50	24.0 ± 1.9 a	68.2 ± 1.1 c	46.0 ± 0.8 ab	74.1 ± 0.8 b

[a] Values in the same column followed by different letters are significantly different ($p < 0.05$). Negative values indicate prooxidant activity.

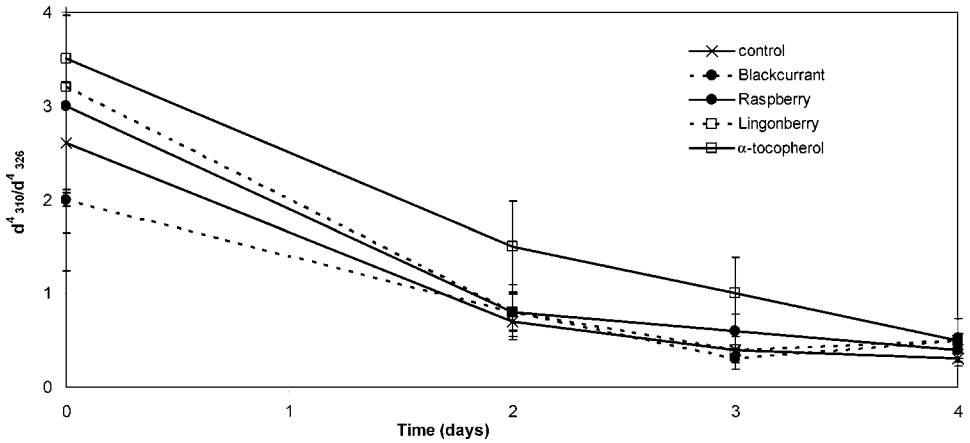

Figure 1. Ratio of fourth-derivative tryptophan emission spectra at 310 and 326 nm during oxidation of emulsions in the absence or presence of 500 μg of blackcurrant, raspberry, and lingonberry anthocyanins per gram or 50 μg of α-tocopherol per gram.

Table 5. Partitioning of Berry Anthocyanins in Emulsion and in a 2% Protein Solution at a Concentration of 500 μg/g (Percent Mean in Water Phase ± Standard Deviation)[a]

anthocyanin fraction	10% emulsified rapeseed oil with 2% whey proteins	2% whey protein solution
blackcurrant	72.8 ± 1.7[b]	80.2 ± 1.3 a[b]
raspberry	70.8 ± 1.8	71.9 ± 1.6 b
lingonberry	69.4 ± 1.1[b]	78.1 ± 0.2 a[b]

[a] Values in the same column followed by different letters are significantly different ($p < 0.05$). [b] Partition is significantly different between different models ($p < 0.05$).

of 500 μg/g. Compared to that of the standard compound, α-tocopherol (50 μg/g), the antioxidant activity of berry anthocyanins was only moderate.

Fourth Derivatives of Tryptophan Emission Spectra. The fourth derivatives of tryptophan emission spectra show that more tryptophan residues are located in a hydrophobic environment (310 nm) than in an aqueous environment (326 nm). The ratio d^4_{310}/d^4_{326} decreases during oxidation (**Figure 1**), indicating that more tryptophan residues were found in the aqueous environment than in the hydrophobic environment. There were no significant differences between the control sample and antioxidant samples with respect to the d^4_{310}/d^4_{326} ratio after oxidation for 4 days.

Partitioning of Berry Anthocyanins in Different Phases. Partitioning of berry anthocyanins between different phases of emulsion is shown in **Table 5**. In samples containing 2% whey proteins, 73.3−79.9% of the anthocyanins were in the water phase. The partitioning into the water phase was weaker with raspberry anthocyanins than with lingonberry and blackcurrant anthocyanins. In samples containing 10% emulsified rapeseed oil, there were no significant differences ($p < 0.05$) in partitioning between different phases of different berry anthocyanin fractions. Significantly more lingonberry anthocyanins were found in the water phase in samples containing only 2% whey proteins than in samples with 10% emulsified rapeseed oil with 2% whey proteins. In a water/oil system consisting of 1 part rapeseed oil and 9 parts water, all anthocyanins located in the water phase (data not shown) and no anthocyanins were found to be incorporated into oil droplets.

DISCUSSION

Berry extracts and different phenolic fractions from blackberries, raspberries, blackcurrants, and blueberries have exhibited both prooxidant and antioxidant properties depending on the oxidation models that were used (*23*, *33*, *34*). Anthocyanins, ellagitannins, and procyanidins isolated from lingonberries, raspberries, bilberries, and blackcurrants have been shown to inhibit both protein and lipid oxidation (*23*). In this study, the antioxidant activity of berry anthocyanins increased with an increase in concentration. All anthocyanins isolated from blackcurrants, raspberries, and lingonberries inhibited both protein and lipid oxidation at the highest tested concentration of 500 μg/g, whereas some berry anthocyanins acted as prooxidants at lower tested concentrations toward either protein or lipid oxidation.

The partitioning behavior of anthocyanins shows that the major part is present in the aqueous phase and approximately 20% is associated with the proteins, i.e., in the interfacial oil/water environment as whey proteins act as emulsifiers forming

viscoelastic layers surrounding the oil droplets. The fourth derivative of tryptophan emission spectra indicates that the tryptophan residues in the aqueous environment are more stable than tryptophan residues in the hydrophobic environment. The reaction between aldehydes and the amino groups of proteins occurs in emulsion at the oil−water interface where most of the proteins form viscoelastic layers around the oil droplets. The more the anthocyanins are associated with the proteins, the more they can inhibit formation of carbonyl compounds. Also, the initial site of modification is located in the hydrophobic regions of the protein molecule, as the fourth-derivative tryptophan spectra show. Thus, it can be concluded that anthocyanins in the aqueous phase inhibit protein and especially tryptophan oxidation. However, the total loss of tryptophan fluorescence indicates that more anthocyanins should be associated with proteins in the interfacial environment and with the oil droplets for better antioxidant activity. It is also possible that some anthocyanins complex copper that is used to initiate oxidation (*35*), and therefore, their antioxidant activity is less toward protein and lipid oxidation.

Blackcurrant anthocyanins were the most potent antioxidants at all tested concentrations compared to raspberry and lingonberry anthocyanins toward oxidation of emulsion. Blackcurrant and raspberry anthocyanins have been reported to exhibit superior antioxidant activity toward oxidation of lactalbumin liposomes (*23*) and oxidation of emulsified methyl linoleate (*28*). Blackcurrant contains delphinidin and cyanidin 3-glucosides and 3-rutinosides, which were shown in this study to be very potent antioxidants in inhibiting both protein and lipid oxidation. In addition, pelargonidin 3-glucoside, present in raspberries, was one of the most efficient anthocyanins toward lipid oxidation. Lingonberry anthocyanins are composed of only different cyanidin glycosides. Obviously, the combination of cyanidin glycosides with either pelargonidin (raspberry) or delphinidin glycosides (blackcurrant) enhanced the antioxidant activity toward both protein and lipid oxidation, although cyanidin glycosides alone were very potent antioxidants at a concentration of 200 μM. Most anthocyanins more strongly inhibited the formation of secondary oxidation products, hexanal and protein carbonyl compounds, than primary oxidation products, conjugated diene hydroperoxides, and loss of tryptophan fluorescence.

The antioxidant activity of berry anthocyanin fractions toward oxidation of emulsion was lower than the antioxidant activity of the control antioxidant sample, α-tocopherol (50 μg/g). This contradicts our earlier findings where anthocyanins have shown to be as potent antioxidants toward protein and lipid oxidation in lactalbumin/liposome model system than α-tocopherol (*23*). However, in liposomes, there are some tocopherols (1.6 ppm) present, and thus, the antioxidant activity may in fact be due to a synergistic effect of tocopherols and anthocyanins. Berry anthocyanins also inhibited protein and lipid oxidation to a greater extent in liposomes (*23*) than in emulsions as shown in this study. It may also be that in liposomes anthocyanins are more closely associated with the site of oxidation compared to their partitioning behavior in whey protein emulsions.

In conclusion, berries rich in antioxidative compounds would have a beneficial impact on the oxidative stability of lipid- and protein-containing food products. However, when berries are added in foods, the total effect of phenolic compounds should be considered because at some concentrations phenolic compounds may not act beneficial. The composition of a food product contributes to the antioxidant activity by affecting of the partitioning of the anthocyanins. Blackcurrant anthocyanins exhibited antioxidant potential most likely due to the presence of cyanidin and delphinidin glycosides.

LITERATURE CITED

(1) Damodaran, S.; Anand, K. Sulfhydryl-disulfide interchange-induced interparticle protein polymerization in whey protein-stabilized emulsions and its relation to emulsion stability. *J. Agric. Food Chem.* **1997**, *45*, 3813−3820.

(2) Monahan, F. J.; McClements, D. J.; Kinsella, J. E. Polymerization of whey proteins in whey protein-stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826−1829.

(3) Færgemand, M.; Otte, J.; Qvist, K. B. Cross-linking of whey proteins by enzymatic oxidation. *J. Agric. Food Chem.* **1998**, *46*, 1326−1333.

(4) Gerrard, J. A.; Brown, P. K.; Fayle, S. E. Maillard crosslinking of food proteins I: The reaction of glutaraldehyde, formaldehyde and glyceraldehydes with ribonuclease. *Food Chem.* **2002**, *79*, 343−349.

(5) Karel, M.; Schaich, K.; Roy, R. B. Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J. Agric. Food Chem.* **1975**, *23*, 159−163.

(6) Howell, N. K.; Herman, H.; Li-Chan, E. C. Y. Elucidation of protein−lipid interactions in lysozyme−corn oil system by Fourier transform Raman spectroscopy. *J. Agric. Food Chem.* **2001**, *49*, 1529−1533.

(7) Leaver, J.; Law, A. J. R.; Brechany, E. Y.; McCrae, C. H. Chemical changes in β-lactoglobulin structure during ageing of protein-stabilized emulsions. *Int. J. Food Sci. Technol.* **1999**, *34*, 503−508.

(8) Leaver, J.; Law, A. J. R.; Brechany, E. Y. Covalent modification of emulsified β-casein resulting from lipid peroxidation. *J. Colloid Interface Sci.* **1999**, *210*, 207−214.

(9) Hunt, J. A.; Dalgleish, D. G. Effect of pH on the stability and surface composition of emulsions made with whey protein isolate. *J. Agric. Food Chem.* **1994**, *42*, 2131−2135.

(10) Fomuso, L. B.; Corredig, M.; Akoh, C. C. Effect of emulsifier on oxidative properties of fish oil-based structured lipid emulsions. *J. Agric. Food Chem.* **2002**, *50*, 2957−2961.

(11) Hu, M.; McClements, D. J.; Decker, E. A. Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *J. Agric. Food Chem.* **2003**, *51*, 1435−1439.

(12) Britten, M.; Giroux, H. J. Interfacial properties of milk protein-stabilized emulsions as influenced by protein concentration. *J. Agric. Food Chem.* **1993**, *41*, 1187−1191.

(13) Diaz, M.; Dunn, C. M.; McClements, J.; Decker, E. A. Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *J. Agric. Food Chem.* **2003**, *51*, 2365−2370.

(14) Hu, M.; McClements, D. J.; Decker, E. A. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate and soy protein isolate. *J. Agric. Food Chem.* **2003**, *51*, 1696−1700.

(15) Lethuaut, L.; Métro, F.; Genot, C. Effect of droplet size on lipid oxidation rates of oil-in-water emulsions stabilized by protein. *J. Am. Oil Chem. Soc.* **2002**, *79*, 425−430.

(16) Viljanen, K.; Kivikari, R.; Heinonen, M. Protein−lipid interactions during liposome oxidation with added anthocyanin and other phenolic compounds. *J. Agric. Food Chem.* **2004**, *52*, 1104−1111.

(17) Decker, E. A.; Ivanov, V.; Zhu, B.-Z.; Frei, B. Inhibition of low-density lipoprotein oxidation by carnosine and histidine. *J. Agric. Food Chem.* **2001**, *49*, 511−516.

(18) Stadtman, E. R.; Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* **1997**, *10*, 485−494.

(19) Van Buren, J. P.; Robinson, W. B. Formation of complexes between protein and tannic acid. *J. Agric. Food Chem.* **1969**, *17*, 772−777.

(20) De Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940−945.

(21) Rawel, H. M.; Kroll, J.; Hohl, U. C. Model studies on reaction of plant phenols with whey proteins. *Nahrung* **2001**, *45*, 72−81.
(22) Heinonen, M.; Rein, D.; Satue-Gracia, M. T.; Huang, S.-W.; German, J. B.; Frankel, E. N. Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. *J. Agric. Food Chem.* **1998**, *46*, 917−922.
(23) Viljanen, K.; Kivikari, R.; Heinonen, M. Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *J. Agric. Food Chem.* **2004**, *52*, 7419−7424.
(24) Schwarz, K.; Frankel, E. N.; German, J. B. Partition behaviour of antioxidative phenolic compounds in heterophasic systems. *Fett/Lipid* **1996**, *98*, 115−121.
(25) Huang, S.-W.; Frankel, E. N.; Aeschbach, R.; German, J. B. Partition of selected antioxidants in corn oil−water model system. *J. Agric. Food Chem.* **1997**, *45*, 1991−1994.
(26) Pekkarinen, S. S.; Stöckmann, H.; Schwarz, K.; Heinonen, I. M.; Hopia, A. I. Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate. *J. Agric. Food Chem.* **1999**, *47*, 3036−3043.
(27) Lampi, A.-M.; Hopia, A.; Ekholm, P.; Piironen, V. Method for the preparation of triacylglycerol fractions from rapeseed and other oils for autoxidation studies. *Lebensm.-Wiss. -Technol.* **1992**, *25*, 386−388.
(28) Kähkönen, M. P.; Heinämäki, J.; Ollilainen, V. Berry anthocyanins: Isolation, identification and antioxidant activity. *J. Sci. Food Agric.* **2003**, *83*, 1403−1411.
(29) Haila, K.; Heinonen, M. Action of β-carotene on purified rapeseed oil during light storage. *Lebensm.-Wiss. -Technol.* **1994**, *27*, 573−577.
(30) Frankel, E. N.; Huang, S.-W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: Bulk oil *vs* emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.
(31) Giessauf, A.; Steiner, E.; Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E independent process during copper-mediated oxidation of LDL. *Biochim. Biophys. Acta* **1995**, *1256*, 221−232.
(32) Rampon, V.; Lathuaut, L.; Mouhous-Riou, N.; Genot, C. Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by front-surface fluorescence. *J. Agric. Food Chem.* **2001**, *49*, 4046−4051.
(33) Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597−3604.
(34) Matsumoto, H.; Nakamura, Y.; Hirayama, M.; Yoshiki, Y.; Okubo, K. Antioxidant activity of blackcurrant anthocyanin aglycons and their glucosides measured by chemiluminescence in a neutral pH region and in human plasma. *J. Agric. Food Chem.* **2002**, *50*, 5034−5037.
(35) Satué-Gracia, M. T.; Heinonen, M.; Frankel, E. N. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *J. Agric. Food Chem.* **1997**, *45*, 3362−3367.

Received for review December 2, 2004. Revised manuscript received January 20, 2005. Accepted January 24, 2005.

JF047975D