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Protein−Lipid Interactions during Liposome Oxidation with Added Anthocyanin and Other Phenolic Compounds

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Oxidation of bovine serum albumin, casein, and lactalbumin and the effect of different procyanidins, anthocyanins, and their aglycons (10 and 20 μM) on lactalbumin oxidation were investigated in a liposome system. Samples were incubated in the dark at 37 °C with copper, and the extent of oxidation was measured by determining the loss of tryptophan fluorescence and the formation of protein carbonyls, conjugated diene hydroperoxides, and hexanal. The correlation between different protein and lipid oxidation measurements was good and statistically significant. Casein was the most stable protein in the liposome model, and it was also the best inhibitor of liposome oxidation. All tested anthocyanins and other phenolic compounds inhibited both lipid and protein oxidation. There were no systematic differences with anthocyanins and their aglycons in relation to the concentrations used or glycosylation with either glucose or rutinose. Procyanidins B1 and B2 and ellagic acid were potentially better antioxidants than anthocyanins due to their several hydroxyl groups as measured by both protein and lipid oxidation. In conclusion, oxidative deterioration of liposomes due to protein−lipid interaction is inhibited by anthocyanins, procyanidins, and ellagitannin present, for example, in berries.

KEYWORDS: Protein oxidation; tryptophan; protein carbonyls; anthocyanins; procyanidins; antioxidants; phenolic compounds

INTRODUCTION

Interactions between lipids and proteins have a significant effect on the progress of oxidative reactions in foods. Due to strong interactions between lipids and proteins in foods the oxidation reactions can easily transfer from lipids to proteins. 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, decrease 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) (*1*−*3*). Moreover, some protein oxidation products can be toxic or carcinogenic (*4*−*8*).

Primary lipid oxidation products (hydroperoxides) and secondary lipid oxidation products (aldehydes and ketones) can react with proteins and in that way cause protein oxidation (*1*, *9*−*11*). Protein oxidation occurs via free radical reactions in which peroxyl radicals ($RO^{\bullet}$ or $ROO^{\bullet}$) formed during lipid oxidation can abstract hydrogen atoms from protein molecules (PH) (reaction 1). Consequently, protein radicals are formed ($P^{\bullet}$), and they can in turn create a protein net (P−P) due to cross-linking (reaction 2).

$$RO^{\bullet}/ROO^{\bullet} + PH \rightarrow P^{\bullet} + ROH/ROOH \quad \text{(reaction 1)}$$

$$P^{\bullet} + P^{\bullet} \rightarrow P{-}P \text{ (protein net)} \quad \text{(reaction 2)}$$

It is also postulated that the protein oxidation process can occur via non-covalent complex formation by both electrostatic and hydrophobic attractions between lipid hydroperoxide (ROOH) or secondary lipid oxidation products (mainly aldehydes and ketones, breakdown products of lipid hydroperoxides) and the nitrogen or sulfur centers of reactive amino acid residues of protein (PH) (reaction 3) (*9*, *11*−*13*). In tryptophan the indole ring is susceptible to irreversible oxidation producing, for example, oxindolylalanine that can be further transformed to *N*-formylkynurenine (NFK; **Scheme 1**) (*14*). The protein radical formed in this reaction can also result in the formation of covalent protein−protein cross-linked derivatives as in reaction 2.

$$ROOH + PH \rightarrow [ROOH\text{- - -}HP] \rightarrow RO^{\bullet} + P^{\bullet} + H_2O \quad \text{(reaction 3)}$$

Protein oxidation can also cause lipid oxidation in the presence of metals. Tryptophan oxidation in proteins and in the presence of copper (Cu^{2+}) or some other initiators ($R^{\bullet}$) is postulated to occur by the mechanism shown in **Scheme 1**.

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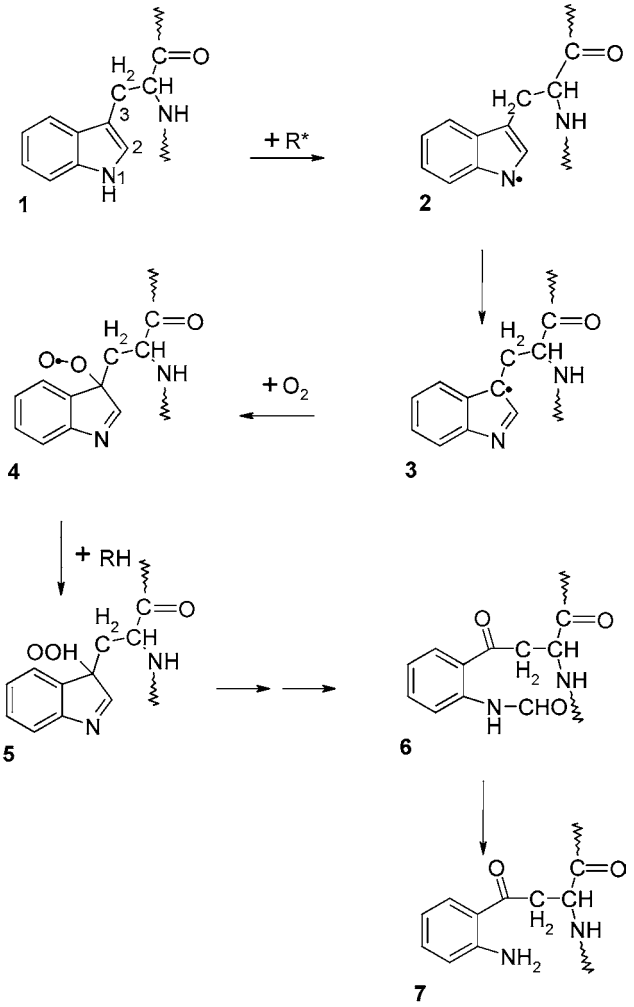
Scheme 1. Formation of *N*-Formylkynurenine and Kynurenine from Tryptophan Residues in Proteins during Oxidation[a]



[a] R* = initiator, RH = fatty acid, 1 = Trp residue, 2 and 3 = free radical forms of Trp residue, 4 = Trp peroxyl radical, 5 = Trp hydroperoxide, 6 = *N*-formylkynurenine, 7 = kynurenine.

Copper can decrease the activation energy of the initiation step of lipid oxidation by directly transferring free radicals to tryptophan residues. First, the initiator converts tryptophan (**1**, Trp) to radicals (**2**, **3**), which can directly react with oxygen (O_2) yielding Trp−peroxyl radicals (**4**). Trp−peroxyl radicals can react with lipids yielding lipid oxidation and Trp−hydroperoxides (**5**, TrpOOH), which can then decompose rapidly to *N*-formylkynurenine (**6**, NFK) and kynurenine (**7**, Kyn) as major end-products (*15*−*18*), carbonyls.

The carbonyl complexes due to protein−lipid interaction are formed rapidly; they are relatively stable and have a specific fluorescence at excitation wavelength around 350 nm. The maximum emission wavelength varies between different carbonyl compounds due to differences in the interacting amino acids and lipid oxidation products (*19*−*22*). Especially lysine residues (which contain free amino groups) in the proteins can form strong fluorescent complexes with aldehydes, resulting in polymerized products typical for nonenzymatic browning (*21*, *23*−*25*). Protein carbonyl compounds are convenient markers of protein oxidation, and they may be involved in cross-linking of damaged proteins via Schiff base formation, which are conjugated fluorophores with distinct spectral properties (excitation wavelength ∼350 nm and emission wavelength ∼450 nm) (*26*).

The most sensitive amino acids toward oxidation are heterocyclic amino acids as well as both the amino and phenolic groups in amino acids. Due to their structures, tryptophan, histidine, and proline, and also lysine, cysteine, and tyrosine, are prone to oxidation where the hydrogen atom is abstracted either from OH-, S-, or N-containing groups (*11*). Oxidation of proteins and amino acids is affected by many environmental factors such as pH, temperature, water activity, and the presence of catalysts or inhibitors. In addition, the three-dimensional structures of each protein affect how proteins can interact with lipids (*27*). For example, casein, due to its disordered random coil and flexible structure, tends to cross-link in the presence of oxidized lipids more readily than the globular and more compact whey proteins (*28*). Proteins and amino acids can also oxidize without lipid radicals by reacting with metals and oxygen.

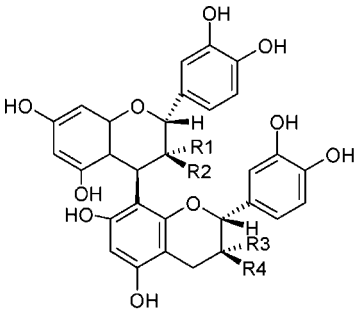
Research on the interactions between food lipids and proteins has mainly focused on the structural aspects. It is well-known that proteins stabilize food emulsions, but they can also act as antioxidants by inhibiting lipid oxidation in membrane model systems such as liposomes (*29*). Especially, histidine residues can act as antioxidants by inhibiting both lipid and protein [low-density lipoprotein (LDL) and bovine serum albumin (BSA)] oxidation (*30*, *31*), but lysine residues have also shown antioxidant activity in lipid oxidation (*30*, *32*). In addition, some BSA oxidation products have been reported to prevent lipid oxidation (*33*, *34*).

Data on the antioxidant activity of phenolic compounds vary widely partly due to the use of different oxidation systems. Phenolic compounds have shown to be effective antioxidants in lipid oxidation (*29*, *35*−*40*). It is postulated that phenolic compounds can inhibit also oxidation of proteins by retarding the oxidation reactions, by binding to the proteins, and by forming complexes between protein molecules. These complexes may inhibit proteins from oxidation (*41*−*43*). Phenolic compounds such as ferulic acid, malvidin, and rutin have been shown to inhibit BSA oxidation in liposome model system (*29*).

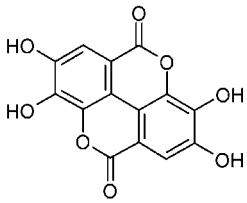
The objectives of this research were to study protein oxidation with different proteins (lactalbumin, casein, and BSA) and their interactions with food lipids and phenolic compounds, especially anthocyanins. It is postulated that the interaction between proteins and lipids has a significant effect on the progress of oxidative reactions in foods. Especially in low-fat food products in which excess fat has often been replaced with protein can the oxidation travel from the lipid phase to the protein phase (*17*, *23*, *27*). Interaction reactions between phenolic compounds, proteins, and lipids are of importance especially in the development of low-calorie functional foods containing increased amounts of phenolic compounds intended for beneficial health effects. Moreover, it has been shown that phenolic compounds improve the food quality (oxidative stability) in inhibiting oxidation of proteins both by binding to the proteins and by retarding the oxidation reactions (*41*−*43*).

MATERIALS AND METHODS

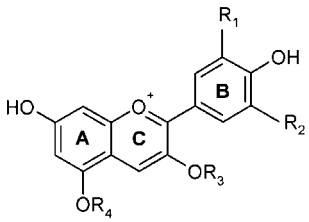
Materials. Procyanidin B1, procyanidin B2, ellagic acid, cyanidin (Cya), delphinidin (Del), pelargonidin (Pel), and their sugars were obtained from Extrasynthèse (Genay, France), except cyanidin 3-(xylosylglucose)-5-galactoside, cyanidin 3-(coumaroyl-xylosylglucose)-5-galactoside, delphinidin 3-glucoside, and delphinidin-3-rutinoside, which were from Polyphenols (Sandnes, Norway). The structures of the phenolic compounds used are shown in **Figure 1**. Lactalbumin, casein, BSA, and L-α-phosphatidylcholine (lecithin from soybean) with a phosphatidylcholine (PC) content of ∼40% were purchased from Sigma Chemical Co. (St. Louis, MO) and copper(II) acetate and α-tocopherol



Procyanidin
B1 R1= OH; R2= H; R3= H; R4= OH
B2 R1= OH; R2= H; R3= OH; R4= H



Ellagic acid



Anthocyanin
Delphinidin R_1= OH; R_2= OH
Cyanidin R_1= OH; R_2= H
Pelargonidin R_1= H; R_2= H
R_3, R_4 = H or sugar moiety

Figure 1. Structures of phenolic compounds used in the study.

Table 1. Fatty Acid Composition of Phosphatidylcholine (PC)

fatty acid	g/100 g	fatty acid	g/100 g
C14:0	0.1	C18:1	12.4
C16:0	19.1	C18:2	57.5
C18:0	3.6	C18:3	7.3

from Merck (Darmstadt, Germany). Ethanol of AAS grade was from Primalco (Rajamaki, Finland), and all other solvents of HPLC grade were from Rathburn Chemicals Ltd. (Walkerburn, Scotland). The citrate buffer was made of citric acid (Pharmia Ltd., Helsinki, Finland) and sodium hydroxide (Dilut-it, J. T. Baker, Deventer, Holland) adjusted to pH 6.6. Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA).

Protein—Liposome Oxidation System. The liposomes were prepared as described by Huang and Frankel (*44*) to a final PC concentration of 0.8 wt %. The liposomes were incubated in the dark at 37 °C with 3 μM cupric acetate for 7 days with 0.16% (i.e., 20% of the PC concentration) lactalbumin, casein, or BSA by using 27.5 mM citrate buffer at pH 6.6. Lactalbumin oxidation in liposomes was also measured in the presence of 10 and 20 μM phenolic compounds. The reference compound, α-tocopherol, was tested only at concentration of 10 μM.

All tested compounds were dissolved in ethanol. Ethanol solutions were pipetted into Erlenmeyer flasks (100 mL), and the solvent was then evaporated with nitrogen. Phosphatidylcholine−protein solution was then added into the flask, and liposomes were prepared by sonicating the solution for 3 min with a U 50 Control Ikasonic sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Germany), and the flasks were sealed before oxidation. The tocopherol content of phosphatidylcholine was investigated by using HPLC (*45*). Phosphatidylcholine contains some α-, γ-, and δ-tocopherols (19, 117, and 59 μg/g, respectively). Also, the fatty acid composition was analyzed by using CG (**Table 1**). The fatty acid composition of phosphatidylcholine reflects the fatty acid composition of soybean oil (*46*). All results of antioxidant studies are given as the mean values of triplicate analyses, and results of protein oxidation studies in liposomes are given as the mean values of duplicate analyses.

Protein Oxidation. Lactalbumin, casein, and BSA oxidation were measured by fluorescence spectroscopy by following both the formation of protein carbonyls and loss of natural tryptophan fluorescence (*17*, *29*). Samples (500 μL) were dissolved in citrate buffer (1 mL). 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 later products of oxidation (protein carbonyls) were recorded from 400 to 500 nm with the excitation wavelength set at 350 nm. For the antioxidant samples the percent inhibition against loss of tryptophan fluorescence was calculated at day 6 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 at day 6 as $[(C_t - S_t)/C_t] \times 100$, where C_t is the fluorescence of protein carbonyls in control sample at time t and S_t is the fluorescence of protein carbonyls in antioxidant sample at time t.

Lipid Oxidation. Lipid (liposome) oxidation was followed by formation of conjugated diene hydroperoxides and formation of hexanal. Samples (50−100 μL) were dissolved in methanol (5 mL), and conjugated diene hydroperoxides were analyzed spectrophotometrically at 234 nm (Lambda Bio UV−vis spectrophotometer, Perkin-Elmer, Norwalk, CT). Hexanal (samples of 500 μL) was measured using static headspace gas chromatography (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. (*47*). The percent inhibition against liposome oxidation was calculated at day 6 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 antioxidant sample at time t.

Statistical Analysis. Differences among antioxidant activities were tested by multivariance analysis using Statgraphics Plus (STCC Inc., Rockville, MD). The significance level was $p < 0.05$. In addition, correlations between different oxidation measurements were calculated.

RESULTS

Liposome Oxidation in the Presence of Different Proteins. All proteins tested, lactalbumin, casein, and BSA, prevented liposomes from oxidizing after the initial lag phase. Casein (76.3 ± 0.01%) and BSA (91.3 ± 0.01%) inhibited conjugated diene hydroperoxides formation better than lactalbumin (18.9 ± 0.52%) measured after 6 days of oxidation (**Figure 2**). Lactalbumin was also the least efficient in inhibiting hexanal formation at the level of 59.2 ± 0.25% compared to casein (98.2 ± 0.07%) and BSA (98.5 ± 0.03%) measured after 6 days of oxidation. The liposomes with and without incorporated proteins were not oxidized in the beginning (before day 3) of the incubation period due to natural tocopherols present in the phosphatidylcholine (1.6 ppm in the final liposome solution). Casein was the most stable protein in the liposome model, as only a 19.0 ± 0.54% decrease in tryptophan fluorescence was detected after 6 days of oxidation, whereas the tryptophan fluorescence of lactalbumin and BSA decreased 44.5 ± 2.18 and 51.0 ± 0.13%, respectively (**Figure 3**). Loss of tryptophan fluorescence is principally due to oxidative changes in the tryptophan residues of the protein. More protein carbonyl compounds were formed in the liposome model with added lactalbumin than in the presence of casein or BSA.

The whey protein, lactalbumin, was obviously less stable than BSA and casein. However, lactalbumin was chosen for further investigations on protein−lipid interactions with added phenolic compounds because of its sensitivity toward oxidation and better

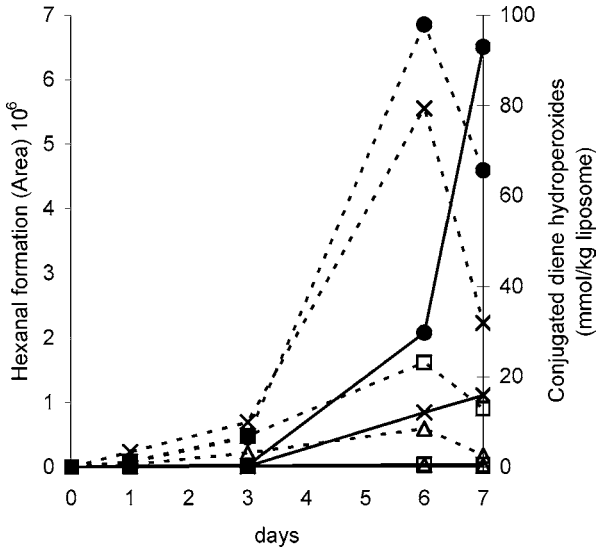


Figure 2. Formation of conjugated diene hydroperoxides (- - -) and hexanal (—) in liposomes (●) in the absence or presence of lactalbumin (×), casein (□), or BSA (△).

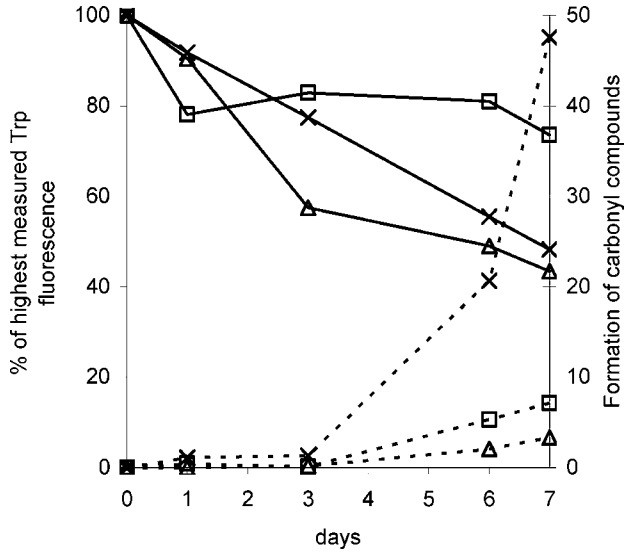


Figure 3. Protein [lactalbumin (×), casein (□), BSA (△)] stability toward oxidation in liposomes measured as loss of tryptophan fluorescence (—) and formation of protein carbonyl compounds (- - -).

applicability for the overall purpose of developing dairy products with phenolic compounds present, for example, in berries. After 3 days of oxidation, the tryptophan fluorescence in lactalbumin was decreased by 22.6 ± 5.7% without any added antioxidants, and at the end of oxidation (7 days) the tryptophan fluorescence was decreased by 51.8 ± 2.3%. Conjugated diene hydroperoxides started to decompose to yield hexanal and other aldehydes and ketones at day 3. At the same time, protein carbonyl compounds were beginning to form from lipid oxidation products and protein amino groups.

The correlations between different lipid and protein oxidation measurements were good and statistically significant. The correlation between formation of carbonyl compounds and formation of hexanal was best ($r = 1.00$, $r^2 = 100\%$, $p = 0.01$). The correlations between loss of tryptophan fluorescence and formation of carbonyl compounds ($r = -0.95$, $r^2 = 90\%$, $p = 0.05$), hexanal ($r = -0.95$, $r^2 = 90\%$, $p = 0.05$), or conjugated diene hydroperoxides ($r = -0.99$, $r^2 = 97\%$, $p = 0.01$) and between formation of conjugated dienes and formation of carbonyl compounds ($r = 0.95$, $r^2 = 91\%$, $p = 0.05$) or hexanal ($r = 0.96$, $r^2 = 91\%$, $p = 0.05$) were also good.

Table 2. Inhibition of Lipid Oxidation (after 6 Days of Oxidation) in Lactalbumin–Lecithin Liposome Oxidation with 10 and 20 μM Anthocyanins and Other Phenolic Compounds (Percent Inhibition, Mean ± SD)[a]

	hydroperoxides		hexanal	
compound[b]	10 μM	20 μM	10 μM	20 μM
Cya	64.5 ± 6.4 bc*	79.6 ± 0.7 bc*	96.1 ± 1.1 a	95.7 ± 0.5 a
Cya-3-glu	79.4 ± 4.4 a	81.2 ± 1.2 b	97.7 ± 1.1 a	96.6 ± 0.2 a
Cya-3,5-diglu	65.8 ± 3.3 bc*	81.3 ± 2.0 b*	95.7 ± 2.1 a	99.0 ± 0.2 a
Cya-3-(xylglu)-5-gal	83.1 ± 1.8 a*	65.7 ± 4.3 cd*	97.4 ± 1.3 a	90.5 ± 0.9 ab
Cya-3-(coum-xylglu)-5-gal	76.6 ± 2.5 ab*	60.5 ± 5.9 de*	98.2 ± 1.0 a	88.9 ± 0.0 bc
Cya-3-rut	57.4 ± 5.0 bc*	74.4 ± 1.8 bc*	90.5 ± 1.6 ab	95.6 ± 0.3 a
Del	8.6 ± 5.9 d*	57.1 ± 5.3 de*	90.1 ± 3.6 ab	97.9 ± 0.5 a
Del-3-glu	0.6 ± 0.1 d*	40.0 ± 8.6 f*	49.1 ± 2.2 d*	81.8 ± 4.1 c*
Del-3-rut	10.5 ± 2.8 d*	49.7 ± 3.0 ef*	42.8 ± 3.7 d*	92.8 ± 3.2 a*
Pel	80.0 ± 6.1 a	82.4 ± 3.4 b	96.9 ± 2.0 a	98.6 ± 0.9 a
Pel-3-glu	11.7 ± 2.8 d*	50.8 ± 4.4 ef*	79.0 ± 7.3 c*	64.8 ± 6.6 d*
procyanidin B1	83.1 ± 0.9 a*	89.2 ± 0.7 a*	76.8 ± 0.1 c	77.9 ± 0.4 c
procyanidin B2	84.1 ± 0.8 a	86.0 ± 3.4 ab	76.4 ± 1.0 c	79.3 ± 2.8 c
ellagic acid	79.2 ± 3.0 ab*	90.8 ± 1.0 a*	76.4 ± 0.1 c	78.9 ± 0.3 c
α-tocopherol	85.4 ± 2.5 a		98.3 ± 1.5 a	

[a] SD, standard deviation. Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$). *, antioxidant activity is significantly different between different concentrations ($p < 0.05$). [b] Cya, cyanidin; Del, delphinidin; Pel, pelargonidin; gal, galactoside; glu, glucoside; diglu, diglucoside; rut, rutinoside; xyl, xylosyl; coum, coumaroyl.

Effect of Phenolic Compounds on Lipid Oxidation in the Lactalbumin—Liposome System. All tested phenolic compounds inhibited both lipid and protein oxidation. Pelargonidin and cyanidin were better inhibitors of the formation of conjugated diene hydroperoxides than delphinidin at both concentrations (**Table 2**). Glycosylation of anthocyanidin with either glucose or rutinose altered the activity order to cyanidin being more active than pelargonidin and delphinidin as measured by the formation of conjugated diene hydroperoxides or hexanal except at the 20 μM addition level pelargonidin-3-glucoside was the weakest antioxidant as measured by the formation of hexanal. In comparison with the aglycons, the activity of glycosides either increased (cyanidin at 10 μM level), remained unchanged (cyanidin at 20 μM level), or decreased (pelargonidin and delphinidin at both concentrations) as measured by the formation of conjugated diene hydroperoxides. There were no statistically significant differences between different anthocyanins and their aglycons, within the concentrations used as measured by the formation of hexanal with the exception of delphinidin 3-glucoside and delphinidin 3-rutinoside being the weakest antioxidants at a concentration of 10 μM. Compared to delphinidin 3-rutinoside, cyanidin-3-rutinoside was a more effective antioxidant at both concentrations.

There were no statistically significant differences in antioxidant activity between different concentrations of procyanidins B1 and B2 and ellagic acid as measured by the formation of conjugated diene hydroperoxides or hexanal. Procyanidins B1 and B2 and ellagic acid inhibited similarly the formation of conjugated diene hydroperoxides and hexanal at both concentrations used. Compared to anthocyanins and their aglycons, procyanidins and ellagic acid were more effective, especially in inhibiting primary lipid oxidation products. The reference compound, α-tocopherol, prevented the formation of conjugated diene hydroperoxides more than most of the anthocyanins and their aglycons, whereas its antioxidant activity was not significantly different from that of the procyanidins and ellagic acid. Toward the formation of hexanal, the antioxidant activity of

Table 3. Inhibition of Protein Oxidation (after 6 Days of Oxidation) in Lactalbumin–Lecithin Liposome Oxidation with 10 and 20 μM Anthocyanins and Other Phenolic Compounds (Percent Inhibition, Mean ± SD)[a]

	tryptophan loss		carbonyl gain	
compound[b]	10 μM	20 μM	10 μM	20 μM
Cya	43.4 ± 0.6 d	49.5 ± 2.0 bc	58.6 ± 0.6 e*	83.9 ± 1.6 bc*
Cya-3-glu	56.8 ± 0.5 b*	46.0 ± 0.7 bc*	90.9 ± 3.5 a	85.0 ± 1.5 ab
Cya-3,5-diglu	34.2 ± 0.9 ef	38.2 ± 2.3 de	79.3 ± 1.6 bc*	91.9 ± 1.1 a*
Cya-3-(xylglu)-5-gal	26.5 ± 1.4 fg*	42.6 ± 0.6 c*	79.1 ± 3.8 bc	72.4 ± 1.5 d
Cya-3-(coum-xylglu)-5-gal	37.6 ± 1.2 de	43.0 ± 1.7 c	82.7 ± 0.7 b	84.6 ± 0.2 ab
Cya-3-rut	33.3 ± 2.8 f	40.8 ± 3.8 cd	81.0 ± 0.6 b	86.2 ± 0.4 ab
Del	19.9 ± 2.6 gh*	54.7 ± 3.4 b*	76.2 ± 2.7 cd*	87.0 ± 0.8 ab*
Del-3-glu	11.7 ± 3.3 h*	29.7 ± 4.5 ef*	39.0 ± 4.2 f*	68.4 ± 3.5 d*
Del-3-rut	12.0 ± 3.0 h*	27.2 ± 4.2 f*	39.2 ± 2.9 f*	80.5 ± 2.4 c*
Pel	53.6 ± 4.9 bc	49.2 ± 1.5 bc	82.9 ± 1.5 ab	83.5 ± 0.9 bc
Pel-3-glu	20.6 ± 3.5 g*	32.9 ± 1.4 def*	71.3 ± 3.8 cd	67.5 ± 4.4 d
procyanidin B1	50.0 ± 3.6 bc	45.9 ± 5.5 bc	76.5 ± 1.4 bc*	34.4 ± 1.3 e*
procyanidin B2	64.3 ± 3.3 a	61.4 ± 8.9 ab	77.1 ± 2.5 bc	85.8 ± 3.2 ab
ellagic acid	55.6 ± 1.9 b	67.0 ± 2.5 a	82.2 ± 1.2 ab*	93.0 ± 1.8 a*
α-tocopherol	62.9 ± 3.6 a		85.3 ± 3.8 ab	

[a] SD, standard deviation. Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$). *, antioxidant activity is significantly different between different concentrations ($p < 0.05$). [b] Cya, cyanidin; Del, delphinidin; Pel, pelargonidin; gal, galactoside; glu, glucoside; diglu, diglucoside; rut, rutinoside; xyl, xylosyl; coum, coumaroyl.

α-tocopherol was comparable to that of pelargonidin, cyanidin, and most cyanidin glycosides.

Effect of Phenolic Compounds on Protein Oxidation in the Lactalbumin—Liposome System. Protein oxidation measured by the loss of tryptophan fluorescence and formation of protein carbonyls was inhibited by anthocyanidins and their aglycons, with delphinidin being more potent than cyanidin and pelargonidin at 20 μM in inhibiting protein oxidation. At 10 μM pelargonidin was the best antioxidant among the anthocyanidins (**Table 3**). Glycosylation of anthocyanidins altered the antioxidant activity order so that cyanidin glycosides were the most effective toward protein oxidation with the exception of cyanidin 3-(xylosylglucose)-5-galactoside at 20 μM in inhibiting protein carbonyl formation. There were no statistically significant differences between pelargonidin 3-glucoside and delphinidin 3-glucoside at the concentration of 20 μM as measured by the loss of tryptophan fluorescence or by the formation of carbonyl compounds. Glycosylation of cyanidin with one sugar (glucose) molecule resulted in better protection against protein oxidation than glycosylation with two or three glucose molecules. The monoglucose derivative of cyanidin was a better antioxidant against protein oxidation compared to the aglycon, with the opposite being true with both delphinidin and pelargonidin. However, compared to the antioxidant activity of α-tocopherol at 10 μM, the anthocyanin aglycons and glycosides were less potent antioxidants. Acylation of cyanidin glycoside with coumaric acid did not improve the oxidative stability of lactalbumin compared to the effect of cyanidin 3-glucoside, although acylation improved the effect of cyanidin 3-(xylosylglucose)-5-galactoside. In comparison with the concentration of 10 μM, the activity of concentrations of 20 μM either decreased (cyanidin 3-glucoside), remained unchanged (cyanidin and pelargonidin), or increased (all other tested compounds) as measured loss of tryptophan fluorescence. There were no statistically significant differences between cyanidin 3-glucoside and -3-rutinoside or between delphinidin 3-glucoside and -3-rutinoside.

Procyanidin B2 (20 μM) inhibited lactalbumin oxidation more than procyanidin B1, with ellagic acid being a more potent antioxidant. There were no statistically significant differences of procyanidins B1 and B2 and ellagic acid antioxidant activity between tested concentrations as measured by the loss of tryptophan fluorescence or carbonyl formation. Procyanidin B1 inhibited significantly more the formation of carbonyl compounds at lower concentration than at higher concentration, whereas procyanidin B2 and ellagic acid were slightly better antioxidants at higher concentration. α-Tocopherol (10 μM) prevented the loss of tryptophan fluorescence as efficiently as procyanidin B2 and the formation of carbonyl compounds as efficiently as ellagic acid, pelargonidin, cyanidin 3-rutinoside, -3-glucoside, and -3-(coumaroyl-xylosylglucose)-5-galactoside.

DISCUSSION

Protein—Lipid Interactions during Liposome Oxidation. Loss of tryptophan fluorescence is an early event of protein oxidation, whereas formation of protein carbonyl compounds is the second sign of protein oxidation due to proteins interacting with secondary lipid oxidation products (*17*, *48*, *49*). In this study the oxidation of different dairy proteins, lactalbumin, casein, and BSA, was investigated by measuring both the loss of tryptophan fluorescence and the formation of protein carbonyl compounds. Tryptophan residues are the sole emitting (330−370 nm) species at the excitation wavelength of 283 nm, and thus the fluorescence method applied in this study provides a specific method to follow oxidative changes in proteins naturally containing only one or a few tryptophan residues. Analytical HPLC methods for analyzing tryptophan oxidation products have been reported (*18*), but they are not applicable to following the oxidation of tryptophan residues in intact proteins. To investigate the protein−lipid interaction, also the influence of proteins on oxidation of liposome phospholipids was measured. The methods used to follow protein oxidation were found to correlate well with methods used to follow the formation of lipid oxidation products such as conjugated diene hydroperoxides and hexanal. This finding reflects the timely interaction reactions of proteins and lipid oxidation products.

Casein, BSA, and lactalbumin all had a stabilizing effect on the liposome oxidation. The three-dimensional structure of proteins affects how proteins can interact with lipids (*27*). Caseins are rather unique in having very little, if any, secondary structure, because of a high level of proline and low sulfuric amino acid content. Possibly it is due to the random coil flexible structure of casein easily unfolding and compensating to the high oxidizability of proline that it was the most stable protein incorporated to liposomes. Casein also was the best inhibitor of phospholipid (liposome) oxidation. Caseins have earlier been reported to be good antioxidants in emulsions partly because they bind copper (*50*, *51*). Especially casein inhibited effectively the formation of hydroperoxides and hexanal (*51*). In the present study casein both itself resisted oxidation, seen as less oxidative damage to tryptophan residues, and inhibited protein−lipid interactions. Fewer lipid oxidation products resulting in carbonyl formation were available in the presence of casein compared to BSA and especially to lactalbumin. BSA was a more effective inhibitor of lipid oxidation than lactalbumin, although lactalbumin was oxidatively more stable measured by the loss of tryptophan fluorescence. Earlier BSA has been reported to have some antioxidant properties in emulsion systems (*52*) and in liposomes, where in the presence of 20% BSA the rates of hydroperoxide and hexanal formation were slower than in the absence of BSA (*29*). BSA is a relatively hydrophobic protein with 17 disulfide bonds adding to its structural stability. In addition, some products of reaction between BSA and primary

and secondary lipid oxidation products and some nonenzymatically browning products of BSA have shown antioxidant activity (*33*, *34*, *53*). The antioxidant activity of BSA and other tested proteins is also directly linked to the ability of their amino acid residues to react with lipid oxidation products. As the amount of tryptophan remains low in all proteins, the protein carbonyls formed also result from lysine, proline, tyrosine, or histidine residues (*54*). Histidine has also been reported to exert antioxidative properties for the prevention of oxidation of tryptophan (*31*).

Giessauf et al. (*17*) reported a tryptophan emission wavelength shift from 353 to 331 nm in LDL oxidation, indicating that most of the tryptophan residues are in hydrophobic environments. Similarly, Burstein et al. (*55*) reported that the tryptophan maximum emission wavelength at 331 nm indicates that most of the tryptophan residues are inside the protein molecule, whereas at 350 nm the tryptophan residues are on the surface of the protein. In this study the tryptophan emission wavelength shifted with lactalbumin and BSA from 340 to 350−360 nm due to oxidation and with casein from 330 to ∼345 nm. This could be interpreted as indicating that most of the tryptophan residues in lactalbumin, casein, and BSA are in the hydrophilic environment, that is, on the surface of the protein molecule and thus exposed to protein−lipid and other interaction reactions.

As to lipid oxidation, the mechanism does not change much in the presence of proteins (*56*). In the present study, the liposome (lipid) oxidation began after a lag phase of 3 days with or without the presence of proteins. This "unaltering" of the lag phase has also been reported previously with BSA (*29*). It seems that proteins do not have an effect on the initial lag period of liposome oxidation reaction. This is due to natural tocopherols present in phosphatidylcholine (PC) originating from the soybean lipid fraction. The commercial lecithin from soybean lipid fractions contains also many other lipids (.∼60%) present in soybean oil, such as triacylglycerols and minor constituents including sterols. The fatty acid profile of the product reflects that of soybean oil, with 12.4% C18:1, 57.5% C18:2, and 7.3% C18:3, thus rendering the lecithin susceptible to oxidation (*46*). It is also notable that all lipids do not have to be oxidized and decomposed before the interaction reactions can take place with amino acid residues of proteins, resulting in the formation of fluorescent protein−carbonyl compounds. For example, in the presence of lactalbumin more lipid oxidation products formed were available for interaction reactions with protein oxidation products also formed in excess compared to the presence of BSA and casein. The formation of carbonyl compounds coincided with the formation of lipid oxidation products at day 3. This is in accordance with earlier reports of BSA and methyl linoleate oxidation products (*56*).

Effect of Antioxidant Phenolic Compounds on Lipid and Protein Oxidation. Phenolic compounds can act as antioxidants by retarding lipid/protein oxidation reaction or by binding to the protein. It is well-known in theory that the antioxidant activity of phenolic compounds is usually enhanced when the number of hydroxyl groups in the B-ring increases. Thus, delphinidin and its glycosides with three hydroxyl groups in the B-ring should have been better antioxidants than cyanidin, pelargonidin, and their glycosides. However, the activity of antioxidants in food and biological systems is dependent on a multitude of factors, including the colloidal properties of the substrate, the conditions and stages of oxidation, and the localization of antioxidants in different phases (*57*). In this study delphinidin inhibited lipid oxidation less than cyanidin and pelargonidin as measured by the formation of conjugated diene hydroperoxides and less than cyanidin as measured by the formation of hexanal. In addition, delphinidin 3-glucoside was a weaker antioxidant than cyanidin and pelargonidin 3-glucosides toward both lipid and protein oxidation. Delphinidin 3-rutinoside inhibited less lipid oxidation than cyanidin 3-rutinoside as well. Possibly delphinidin and its glucosides are more strongly bound to lactalbumin or are forming more complexes with phospholipids than other anthocyanins and their aglycons, thus having only a moderate effect on lipid oxidation. This is supported by earlier findings that contrary to other anthocyanidins delphinidin acts as prooxidant in liposome model system in the presence or absence of BSA (*29*, *35*) but has been reported to effectively inhibit the oxidation of LDL and methyl linoleate emulsions (*35*, *40*). Delphinidin has also been shown to efficiently chelate copper used as an initiator of liposome oxidation (*35*). In LDL oxidation glycosylation of anthocyanins generally resulted in lower activities, whereas in bulk oil the glycosides were more effective than the aglycons (*40*). Pelargonidin was clearly the weakest antioxidant in inhibiting the oxidation of emulsified methyl linoleate, and anthocyanins and their aglycons are relatively ineffective or even prooxidants in bulk methyl linoleate. On the other hand, they work fine in hydrophilic environments such as liposomes, emulsions, and LDL (*29, 40*).

The procyanidin B1 and B2 molecules investigated in this study both contain 10 hydroxyl groups and differ in their structure only in the stereochemical position of the hydroxyl group at C-3. Due to this there were no significant differences between these compounds. Both procyanidins were effective antioxidants in preventing both lipid and protein oxidation. Ellagic acid contains only four hydroxyl groups, but it was a more potent antioxidant in the lactalbumin−liposome system toward protein oxidation and an equal antioxidant toward lipid oxidation compared to procyanidins B1 and B2. There were no statistically significant differences between concentrations used. The antioxidant activity toward conjugated diene hydroperoxide formation of phenolic compounds containing more hydroxyl groups such as procyanidins B1 and B2 and ellagic acid was the same as the antioxidant activity of anthocyanins containing fewer hydroxyl groups such as cyanidin 3-glucoside, -3-(xylosylglucose)-5-galactoside, -3-(coumaroyl-xylosylglucose)-5-galactoside, and pelargonidin. However, cyanidin and its glycosidic forms were more potent antioxidants toward hexanal formation than procyanidins B1 and B2 and ellagic acid. For ellagic acid radical scavenging capacities (*58*, *59*) and moderate antioxidant activities have been reported earlier in bulk and emulsified methyl linoleate (*58*). Also, procyanidins have been shown to be able to scavenge radicals (*59*, *60*).

In conclusion, procyanidin B2 and ellagic acid were the most effective phenolic compounds in inhibiting the loss of tryptophan fluorescence at higher addition level. Delphinidin was not a good antioxidant toward lipid oxidation, although both delphinidin and the procyanidins were powerful antioxidants in preventing tryptophan degradation at a concentration of 20 μM. Protein oxidation was also efficiently retarded by pelargonidin and cyanidin. Foods in which strong interactions occur between proteins and lipids are multiphase foods such as milk and dairy products and cheese, mayonnaise, meat products, and bakery products. Due to the strong interactions the oxidation reactions can easily transfer from lipids to proteins. Thus, phenolic compounds preventing both lipid and protein oxidation may prove to be beneficial both in improving oxidative stability, that is, the shelf life of foods, and in developing functional foods, for example, of dairy origin with added berries. The most

effective phenolic compounds in inhibiting protein−lipid interactions resulting in oxidative deterioration were ellagic acid, pelargonidin, cyanidin 3-glucoside, and the procyanidins, all of which are natural constituents of many plant raw materials, including berries.

ABBREVIATIONS USED

BSA, bovine serum albumin; PC, phosphatidylcholine; Cya, cyanidin; Del, delphinidin; Pel, pelargonidin; gal, galactoside; glu, glucoside; diglu, diglucoside; rut, rutinoside; xyl, xylosyl; coum, coumaroyl.

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