JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Oxidation of Skeletal Muscle Myofibrillar Proteins in Oil-in-Water Emulsions: Interaction with Lipids and Effect of Selected Phenolic Compounds

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The effect of selected phenolic compounds, namely, gallic acid, cyanidin-3-glucoside, (+)-epicatechin, chlorogenic acid, genistein and rutin (50 and 200 μM), and α-tocopherol (50 μM) against the oxidation of oil-in-water emulsions (37 °C/10 days) containing 1% myofibrillar proteins (MPs), was investigated. Emulsions containing 1% bovine serum albumin (BSA) were also prepared for comparative purposes. Protein oxidation was assessed by measuring the loss of natural tryptophan fluorescence and the protein carbonyl gain by using fluorescence spectroscopy. Lipid oxidation was concurrently analyzed by measuring the increase of conjugated dienes (CDs) and hexanal. Proteins inhibited lipid oxidation in oil-in-water emulsions, and MPs showed a more intense antioxidant activity than BSA. MPs were also more resistant to oxidative deterioration than BSA. The different antioxidant capacity of MPs and BSA and their susceptibility to suffer oxidative reactions might be derived from their different amino acid composition and three-dimensional structures. The addition of the phenolic compounds resulted in a variety of effects, including both antioxidant and pro-oxidant effects. Gallic acid, cyanidin-3-glucoside, and genistein were the most efficient inhibitors of lipid and protein oxidation. The chemical structure of the phenolic compounds as well as the nature and conformation of the proteins were greatly influential on the overall effect against oxidative reactions.

KEYWORDS: Myofibrillar proteins; protein carbonyls; tryptophan fluorescence; conjugated dienes; hexanal; phenolics

INTRODUCTION

Myofibrillar proteins (actin and myosin) are the most abundant proteins in muscle foods and play a decisive role in muscle foods regarding sensory, nutritional, and technological aspects (*1*). Muscle foods are considered excellent sources of high-quality protein for the diet because muscle proteins are rich in essential amino acids. The success of the technological processes applied for the manufacture of meat products are supported by the functional properties of myofibrillar proteins. The physicochemical state and integrity of the myofibrillar proteins affects their functionality and, therefore, the quality and value of the processed meat products (*2*). After slaughter of meat animals, *in vivo* antioxidant mechanisms partly collapse and muscles are exposed to lipid and protein oxidation during handling, storage, and industrial processing (*1*).

The oxidation of myofibrillar proteins involves the loss of essential amino acids and the generation of oxidation products, such as cross-links (disulphide bonds and dityrosines), amino-acid-oxidized derivatives, and protein carbonyls (*3*). According to previous studies, the oxidation of myofibrillar proteins leads to the loss of nutritional quality and protein functionality (*4*). Lately, numerous studies have dealt with the development of protein oxidation in muscle foods and have shed light on the influence of meat origin and industrial processing on the occurrence and intensity of protein oxidation (*5*−*7*). The oxidation of myofibrillar proteins during processing and storage of meat products is associated with the loss of quality, leading to texture and color changes (*7, 8*). In contrast, the mechanisms involved in the oxidation of myofibrillar proteins and the interaction with lipids in food systems are poorly understood. Previous mechanistic approaches have mainly focused on the oxidation of isolated myofibrillar proteins through the assessment of conformational changes of the oxidized myosin, the loss of particular amino acids, and the quantification of protein oxidation products, mainly cross-links and DNPH-labeled carbonyls (*9*−*11*). In muscle foods, however, myofibrillar proteins are oxidized in the presence of other oxidizable substrates, such as unsatur-

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10.1021/jf801784h CCC: $40.75
Published on Web 11/01/2008

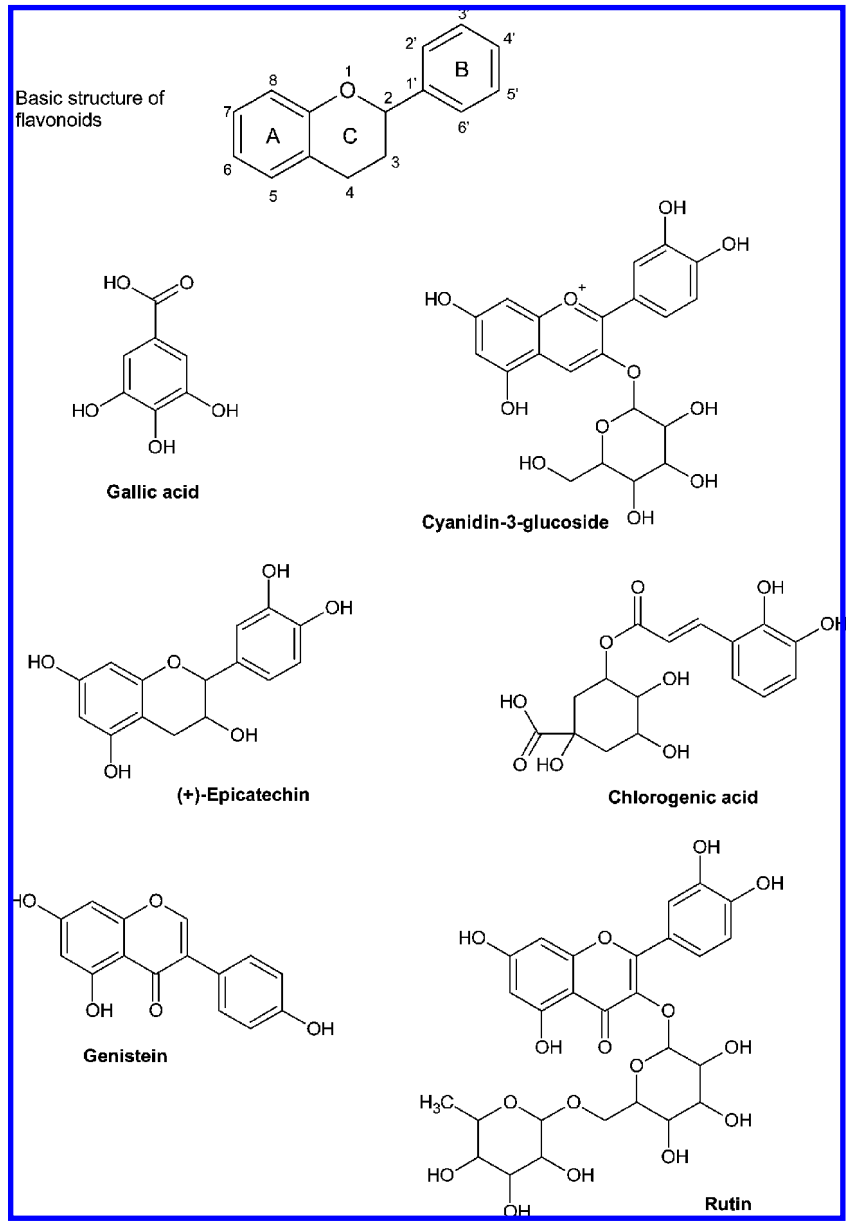

Figure 1. Basic structure of flavonoids and structures of phenolic compounds tested in this study.

ated lipids, with which proteins have been proven to interact (*12*). However, there is no information available regarding the interaction between lipids and myofibrillar proteins during the development of oxidative reactions in meat model systems.

The adverse effects of protein oxidation on muscle foods have challenged meat technologists to develop successful antioxidant strategies. Most studies confirm the increasing interest in natural antioxidants, mainly plant materials containing high levels of phenolic compounds, as inhibitors of protein oxidation in muscle foods. Fruits, vegetables, spices, and leaves have been successfully used as inhibitors of protein oxidation in muscle foods (*6*−*8*). However, there is no information regarding the particular mechanisms through which phenolic compounds protect myofibrillar proteins against oxidative damage. According to previous studies carried out on myoglobin (*13*), bovine serum albumin (BSA) (*14*), and dairy proteins (*12*), phenolic compounds act as radical scavengers and might interact with proteins through the establishment of covalent and noncovalent links. The phenolic−protein interaction might affect the efficiency of the phenolic compounds and is affected by several factors, including the amount and chemical structure of the phenolics (*13*) and the size, conformation, and charge of the protein molecules (*15*). Thus far, plant phenolics have never been evaluated as inhibitors of the oxidation of myofibrillar proteins, and the consequences of their interactions remain unknown.

The present study was aimed to provide information about (i) the susceptibility of myofibrillar proteins to oxidation in oil-in-water emulsions, (ii) the protein−lipid interaction during oxidation development, and (iii) the effect of selected phenolic compounds on myofibrillar protein oxidation.

MATERIALS AND METHODS

Materials. Cyanidin-3-glucoside, caffeoylquinic acid (chlorogenic acid), (+)-epicatechin, rutin, and genistein were purchased from Extrasynthese (Germany, France), while gallic acid was obtained from Sigma Aldrich (Steinheim, Germany). The structures of the phenolic compounds used in the present study are shown in **Figure 1**. BSA was purchased from Sigma Chemical Co. (St. Louis, MO), whereas copper(II) acetate, potassium phosphate, and α-tocopherol were purchased from Merck (Darmstadt, Germany). Methanol and iso-octane of AAS grade were supplied by J.T. Baker (Deventer, Holland) and Riedel dehäen (Seelze, Germany), respectively. The other chemicals

[sodium chloride, ethylene glycol tetraacetic acid (EGTA), and magnesium chloride] were purchased from Sigma Aldrich (Steinheim, Germany). Water used was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA). Rapeseed oil and porcine longissimus dorsi muscle were purchased in local supermarkets in Helsinki, Finland.

Extraction of Myofibrillar Proteins. Myofibrillar proteins (MPs) were extracted from porcine longissimus dorsi muscle according to the procedure used by Park et al. (*11*), with minor modifications. Minced muscle was homogenized for 30 s with 4 vol (v/w) of a cold isolation buffer (10 mM potassium phosphate, 0.1 N NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7). Samples were centrifuged (2000 rpm for 15 min at 4 °C), and the supernatant was discarded, while the pellet was washed twice with 4 vol (v/w) of the same buffer. Then, the myofibrillar pellet was washed 3 times with 4 vol of 0.1 N NaCl. Before the third centrifugation, the myofibrillar suspension was filtered through a gauze, and the pH was adjusted to 6.0 with 0.1 N HCl. After the last centrifugation, the myofibrillar protein isolate (MPI) was stored in a tightly capped bottle at 0 °C and used within 48 h for the production of oil-in-water emulsions. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed to evaluate the purity of the protein extract. The protein concentration was measured following the Lowry method (*16*), and BSA was used as a standard.

Preparation of Emulsions. According to the source of protein (myofibrillar proteins or BSA), two different types of emulsions were considered: MP and BSA emulsions. Before use, both proteins (MPI and BSA) were solubilized in 10 mM potassium phosphate buffer (pH 6) containing 0.6 N sodium chloride. Oil-in-water emulsions (10%, w/w) were prepared following the procedure described by Viljanen et al. (*17*). A total of 1 g of purified rapeseed oil, 0.20 g of protein, and 9 mL of 10 mM potassium phosphate buffer (pH 6) containing 0.6 N sodium chloride were mixed and sonicated for 3 min with a U 50 Control Ikasonic sonicator (Janke and Kunkel GmbH and Co. KG, Staufen, Germany) in an ice bath. Before being used, purified rapeseed oil was checked to be free of tocopherols with a high-performance liquid chromatography (HPLC) method developed by Haila and Heinonen (*18*). Both MP and BSA emulsions were used to prepare eight different emulsions depending upon the addition of selected phenolic compounds (gallic acid, cyanidin-3-glucoside, chlorogenic acid, epicatechin, rutin, and genistein), including emulsions with α-tocopherol and control emulsions (with no phenolic compound). Phenolic compounds (at levels of 50 and 200 μM corresponding to approximately 250 and 1000 μg/g, respectively) 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 emulsions in the sealed vials were oxidized in the dark at 37 °C for 10 days. During the pro-oxidant storage, the emulsions were constantly stirred with magnets, and sampling was carried out at days 1, 3, 5, 7, and 10 for analyses.

Lipid Oxidation. Lipid oxidation was assessed by measuring the levels of primary (conjugated diene hydroperoxides) and secondary (hexanal) oxidation products during storage of samples. Conjugated diene hydroperoxides (CDs) were isolated and analyzed according to the method described by Viljanen et al. (*12*). An aliquot of the emulsion (100 μL) was mixed with 1.5 mL of iso-octane-2-propanol (3:1, v/v) and vortexed (10 s, 3 times). The organic solvent phase (0.2 mL) was obtained after centrifugation at 3500 rpm for 5 min and diluted to 5 mL of iso-octane. Samples were then stirred, and the absorbance was measured at 234 nm with an spectrophotometer (Shimadzu, UV-1601, UV−vis spectrometer). CDs were quantified by using 25 200 M^{-1} cm^{-1} as a molar extinction coefficient.

Hexanal was measured using static headspace gas chromatography (Autosystem XL gas chromatograph equipped with a HS40XL headspace sampler; Perkin-Elmer, Shelton, CT; column NB-54, Nordion) according to the method described by Viljanen et al. (*12*). The percent inhibition against hexanal formation was calculated at day 10 using the following equation: $[(C_{10} - A_{10})/C_{10}] \times 100$, where C_{10} is the level of hexanal in the control sample at day 10 and A_{10} is the count of hexanal in the antioxidant sample at day 10.

Protein Oxidation. The oxidation of myofibrillar proteins and BSA was evaluated by assessing both the loss of natural tryptophan fluorescence and the formation of protein carbonyls in the emulsions using fluorescence spectroscopy (*19, 20*).

Samples (100 μL for tryptophan fluorescence and 250 μL for carbonyl gain) were dissolved in phosphate buffer (5 mL). Emission spectra of tryptophan were recorded from 300 to 400 nm with the excitation wavelength established at 283 nm (LS 55 Perkin-Elmer luminescence spectrometer). Emission spectra of protein carbonyls were recorded from 400 to 500 nm, with the excitation wavelength set at 350 nm. The percent inhibition against the loss of tryptophan and gain of protein carbonyl fluorescence was calculated at day 10 as $[(A_{10} - C_{10})/A_{10}] \times 100$, where C_{10} is the fluorescence of the control sample at day 10 and A_{10} is the fluorescence of the antioxidant sample at day 10.

Yellowness Measurement. The yellowness was determined in 1 mL of emulsion diluted in 5 mL of MilliQ water by using the weighted ordinate method (*21*). Tristimulus values (x, y, and z) were calculated from the transmittances (T) obtained by spectrophotometry from 400 to 700 nm at constants intervals of 10 nm. Transmittance values were then converted into the corresponding Tristimulus and into the CIELAB L^*, a^*, and b^* color values. The yellowness index (YI) at different times was calculated according to Francis and Clydeslade (*21*) as follow: yellowness index $= 142.86b^*/L^*$.

Data Analysis. All types of emulsion were made in triplicate, and all analyses were made in duplicate ($n = 6$). Data obtained from statistical analyses were used as variables and computed in an analysis of variance using SPSS for Windows, version 6.1, to study the effect of the added phenolic compounds. Student's t tests were performed to compare means derived from emulsions with different protein source (MP versus BSA) and different phenolics level (50 versus 200 μM). Pearson correlations were also calculated to establish relationships between parameters. Statistical significance was set at $p < 0.05$.

RESULTS

Emulsion Oxidation in the Presence of MP and BSA. The evolution of CD during oxidation of MP and BSA emulsions is similar to that reported in previous studies (*12*−*22*) (**Figure 2**). According to the results obtained in the present study, the influence of MP on lipid oxidation was different to that shown by BSA. In BSA emulsions, the increase of the amount of CD during the first stages of the oxidation process reached the maximum at day 5 and was followed by a decrease at the end of the storage. The presence of MP caused an increase in the lag time of lipid oxidation because the highest level of CD in MP emulsions was reached later than in BSA emulsions (at day 7). Additionally, MP emulsions contained significantly lower amounts of CD than BSA emulsions at all sampling days, except day 10. Consistently, the headspace (HS) in BSA emulsions contained significantly higher levels of hexanal than that in MP emulsions at all sampling days.

The evolution of the loss of tryptophan fluorescence and the gain of protein carbonyl during storage of MP and BSA emulsions is displayed in **Figure 3**. The tryptophan level decreased in both types of emulsions over time as a likely consequence of the oxidative modification of the amino acid. However, the loss of tryptophan fluorescence was larger in the BSA emulsions than in the MP counterparts. Significant differences were found between emulsions for tryptophan depletion at all sampling times. The fluorescence emitted by protein carbonyls increased over time, with the highest fluorescence being measured at day 10. According to the fluorescence measured, MP emulsions showed, at all sampling days, significantly lower amounts of protein carbonyls than BSA emulsions.

Effect of Phenolics on Lipid and Protein Oxidation. The percent inhibition against lipid oxidation obtained for each

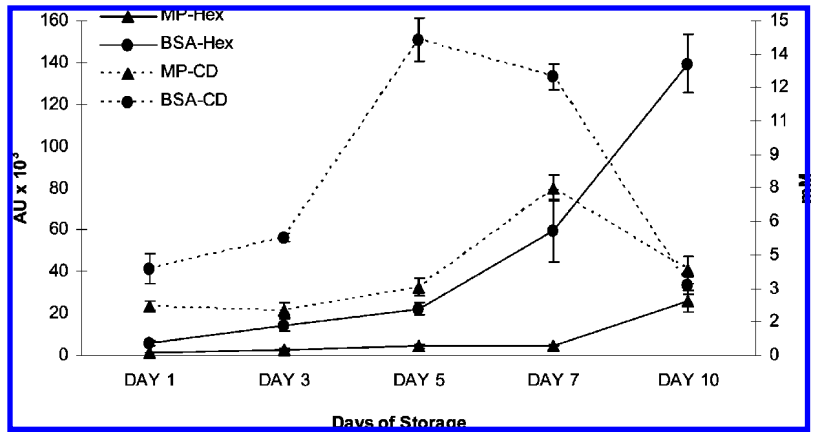

Figure 2. Evolution of lipid oxidation as measured by the formation of CD (mM) and hexanal (Hex) counts (AU $\times$ 10^3) during pro-oxidant storage of MP and BSA emulsions (mean $\pm$ standard deviation).

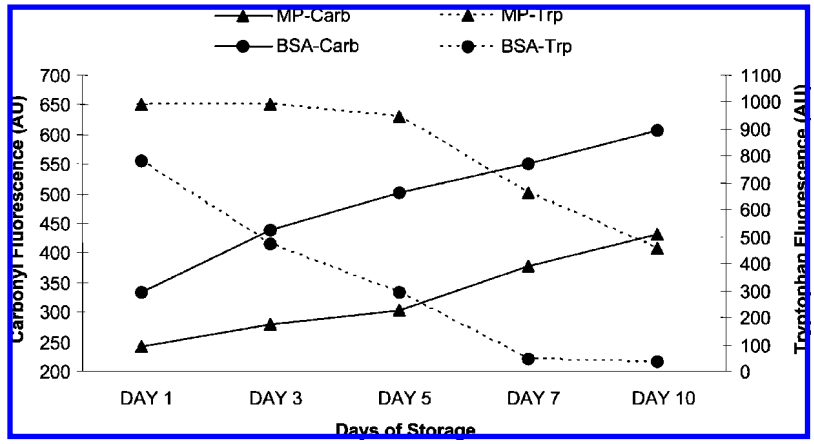

Figure 3. Evolution of tryptophan (Trp) and protein carbonyl fluorescence (Carb) (AU) during pro-oxidant storage of MP and BSA emulsions (mean $\pm$ standard deviation).

Table 1. Inhibition of Hexanal Generation after 10 Days of Pro-oxidant Storage in MP and BSA Emulsions with Added Selected Phenolic Compounds (Percent Inhibition, Mean $\pm$ Standard Deviation)[a]

	MP		BSA	
	50 μM	200 μM	50 μM	200 μM
gallic acid	27.5 $\pm$ 1.3 c[b]	81.0 $\pm$ 4.1 a[b]	31.0 $\pm$ 8.9 c[b]	74.7 $\pm$ 7.5 a[b]
cyanidin-3-glucoside	0.3 $\pm$ 2.7 d[b]	71.9 $\pm$ 1.0 b[b]	38.8 $\pm$ 7.1 c[b]	72.4 $\pm$ 4.0 a[b]
epicatechin	65.5 $\pm$ 7.1 b[b]	−17.7 $\pm$ 3.8 d[b]	38.6 $\pm$ 11.1 c[b]	−0.2 $\pm$ 1.3 c[b]
chlorogenic acid	−2.6 $\pm$ 0.2 d[b]	57.1 $\pm$ 4.9 c[b]	−16.0 $\pm$ 3.4 d[b]	51.9 $\pm$ 12.3 b[b]
genistein	63.4 $\pm$ 6.6 b	78.3 $\pm$ 7.9 ab	52.5 $\pm$ 10.2 b[b]	81.8 $\pm$ 4.0 a[b]
rutin	−20.5 $\pm$ 6.3 e[b]	−76.5 $\pm$ 11.9 e[b]	−15.1 $\pm$ 5.1 d[b]	−36.8 $\pm$ 5.2 d[b]
tocopherol	90.3 $\pm$ 2.6 a		70.5 $\pm$ 3.8 a	

[a] Negative values denote pro-oxidant activity. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Significant differences ($p < 0.05$) between concentrations.

Table 2. Inhibition of Tryptophan Loss after 10 Days of Pro-oxidant Storage in MP and BSA Emulsions with Added Selected Phenolic Compounds (Percent Inhibition, Mean $\pm$ Standard Deviation)[a]

	MP		BSA	
	50 μM	200 μM	50 μM	200 μM
gallic acid	12.8 $\pm$ 3.9 c[b]	25.4 $\pm$ 1.6 a[b]	23.0 $\pm$ 4.3 d[b]	69.1 $\pm$ 6.3 a[b]
cyanidin-3-glucoside	−1.1 $\pm$ 6.56 d[b]	25.7 $\pm$ 2.9 a[b]	32.8 $\pm$ 3.8 c[b]	53.0 $\pm$ 7.8 b[b]
epicatechin	14.5 $\pm$ 2.8 bc[b]	−17.8 $\pm$ 5.1 b[b]	47.5 $\pm$ 5.0 b[b]	3.2 $\pm$ 3.1 d[b]
chlorogenic acid	−6.3 $\pm$ 3.9 d[b]	22.3 $\pm$ 3.3 a[b]	−8.1 $\pm$ 4.5 e[b]	18.3 $\pm$ 2.8 c[b]
genistein	20.6 $\pm$ 5.2 b	21.5 $\pm$ 4.3 a	42.9 $\pm$ 6.1 b	47.2 $\pm$ 6.3 b
rutin	−5.9 $\pm$ 2.5 d[b]	−68.1 $\pm$ 13.8 c[b]	−1.6 $\pm$ 5.6 e	−12.4 $\pm$ 5.5 e
tocopherol	43.7 $\pm$ 2.4 a		72.8 $\pm$ 5.7 a	

[a] Negative values denote pro-oxidant activity. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Significant differences ($p < 0.05$) between concentrations.

selected phenolic compound at day 10 is shown in **Table 1**. Except rutin, all tested phenolic compounds exhibited an antioxidant effect against the generation of hexanal. Four compounds, namely, gallic acid, cyanidin-3-glucoside, chlorogenic acid, and genistein, showed a clear antioxidant effect, and this effect was more intense at higher concentrations of the phenolic compound. The epicatechin showed an opposite behavior because the highest inhibition was found at 50 μM, whereas a weak pro-oxidant effect (nonsignificant) was observed at 200 μM. Rutin showed a pro-oxidant effect, with that being more intense at the higher concentration of the phenolic compound. The reference compound (α-tocopherol) prevented hexanal formation to a higher extent than tested phenolic compounds, even when these were added at a higher concentration. The effect of phenolic compounds was similar in both MP and BSA emulsions. Among phenolic compounds, gallic acid, genistein, and cyanidin-3-glucoside showed the highest percent inhibitions (above 70%) in both types of emulsions.

Phenolic compounds displayed different effects against protein oxidation. In agreement with results obtained in the present study for lipid oxidation, gallic acid, cyanidin-3-glucoside, chlorogenic acid, and genistein inhibited tryptophan depletion to a significant extent when added at 200 μM (**Table 2**). In contrast, epicatechin only showed a significant antioxidant effect at a lower concentration (50 μM). Rutin showed no effect when added at 50 μM, and a potent pro-oxidant effect was observed at higher concentrations. According to the results

Table 3. Inhibition of Carbonyl Gain after 10 Days of Pro-oxidant Storage in MP and BSA Emulsions with Added Selected Phenolic Compounds (Percent Inhibition, Mean ± Standard Deviation)[a]

	MP		BSA	
	50 μM	200 μM	50 μM	200 μM
gallic acid	12.6 ± 2.9 bc	21.5 ± 4.6 b	14.2 ± 3.1 b[b]	23.6 ± 3.2 bc[b]
cyanidin-3-glucoside	19.4 ± 4.4 b[b]	31.3 ± 4.3 a[b]	12.3 ± 3.8 b[b]	30.3 ± 6.5 ab[b]
epicatechin	12.4 ± 3.1 bc[b]	−6.9 ± 0.8 d[b]	13.8 ± 2.3 b[b]	−5.0 ± 1.6 d[b]
chlorogenic acid	1.4 ± 3.2 cd[b]	10.9 ± 2.2 c[b]	−6.3 ± 3.1 c[b]	18.0 ± 1.8 c[b]
genistein	26.6 ± 5.0 b	39.3 ± 10.4 a	15.3 ± 2.9 b[b]	30.9 ± 4.9 a[b]
rutin	−3.4 ± 3.3 d	−8.0 ± 3.0 d	−4.1 ± 1.7 c[b]	−11.4 ± 2.8 d[b]
tocopherol	49.3 ± 10.4 a		37.8 ± 1.3 a	

[a] Negative values denote pro-oxidant activity. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Significant differences ($p < 0.05$) between concentrations.

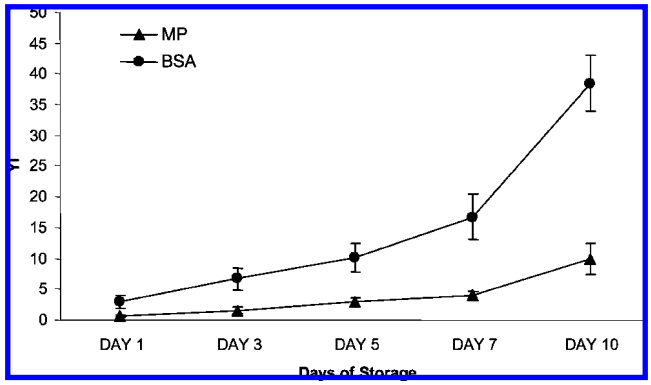

Figure 4. Evolution of YI during pro-oxidant storage of MP and BSA emulsions (mean ± standard deviation).

obtained, the effect of phenolic compounds was influenced by the proteins because the antioxidant effect of some particular compounds, namely, gallic acid, cyanidin-3-glucoside, genistein (200 μM), and epicatechin (50 μM), was higher in BSA emulsions than in MP emulsions. Again, α-tocopherol showed the highest inhibitory effect against tryptophan oxidation, and this effect was also more intense in BSA emulsions than in the MP counterparts.

The percent inhibition displayed by selected phenolic compounds against protein carbonyl generation in MP and BSA emulsions is shown in **Table 3**. In accordance with the above-mentioned results for tryptophan loss, gallic acid, cyanidin-3-glucoside, and genistein showed an inhibitory effect against carbonyl generation at 200 μM. Chlorogenic acid (200 μM) and epicatechin (50 μM) also displayed an inhibitory effect but not to a significant extent. Once more, rutin showed a slight pro-oxidant effect. The highest percent inhibition against protein carbonyl formation was obtained for α-tocopherol. In this case, the role played by phenolic compounds was similar in both MP and BSA emulsions. In general, the percent inhibitions displayed by phenolics against protein oxidation were lower than those displayed against lipid oxidation.

During the oxidation process, the color displayed by emulsions turned from a milky-white color to a yellowish one, and this color evolution was recorded spectrophotometrically as a YI (**Figure 4**). In agreement with the oxidation measurements, MP emulsions showed significantly lower YI at all sampling times than BSA emulsions. Most phenolic compounds inhibited the generation of the yellowish color, whereas rutin (200 μM) promoted the yellowness. In general, the percent inhibitions found against yellowness development was similar to those found against lipid oxidation (**Table 4**). In both MP and BSA emulsions, gallic acid, cyanidin-3-glucoside, and genistein showed the highest inhibitory effect (around 80%).

Table 4. Inhibition of Yellowness after 10 Days of Pro-oxidant Storage in MP and BSA Emulsions with Added Selected Phenolic Compounds (Percent Inhibition, Mean ± Standard Deviation)[a]

	MP		BSA	
	50 μM	200 μM	50 μM	200 μM
gallic acid	44.3 ± 9.2 c[b]	84.4 ± 3.2 a[b]	54.2 ± 9.0 c[b]	82.2 ± 4.7 a[b]
cyanidin-3-glucoside	5.4 ± 2.2 d[b]	77.0 ± 3.6 ab[b]	39.6 ± 10.9 c[b]	80.4 ± 2.6 a[b]
epicatechin	72.5 ± 4.2 b[b]	−17.4 ± 4.1 c[b]	78.6 ± 3.2 b[b]	3.2 ± 1.0 b[b]
chlorogenic acid	6.5 ± 1.7 d[b]	68.5 ± 6.9 b[b]	35.5 ± 13.4 c[b]	75.5 ± 5.4 a[b]
genistein	70.8 ± 4.4 b[b]	82.2 ± 3.3 a[b]	73.4 ± 4.2 b	74.3 ± 4.8 a
rutin	−16.3 ± 5.8 e[b]	−57.5 ± 27.1 c[b]	2.3 ± 0.9 d[b]	−22.2 ± 12.6 c[b]
tocopherol	91.7 ± 2.4 a		87.2 ± 3.8 a	

[a] Negative values denote pro-oxidant activity. Means in the same column with different letters are significantly different ($p < 0.05$). [b] Significant differences ($p < 0.05$) between concentrations.

DISCUSSION

Emulsion Oxidation in the Presence of MP and BSA. The proteins used in the present study had a stabilizing effect on the emulsions. The antioxidant potential of BSA and other proteins have been profusely described in the literature. Viljanen et al. (*12*) reported the ability of BSA, casein, and other dairy proteins to inhibit the formation of hydroperoxides and hexanal in liposomes. Heinonen et al. (*20*) and Lethuaut et al. (*23*) described the antioxidant properties of BSA in emulsion systems and liposomes, respectively. According to the results from previous studies (*20*), MP also exhibit a clear protective role against lipid oxidation in oil-in-water emulsions. The antioxidant properties of proteins are attributed to the cooperative effect of a variety of properties, including the ability of aromatic and sulfur-containing amino acids, such as proline and methionine, to scavenge free radicals and the capacity to act as metal-ion chelators (*12, 24, 25*). Particularly, the antioxidant activity of BSA is mainly attributed to the ability of certain amino acids to scavenge lipid-derived radicals. Additionally, some non-enzymatic browning products of BSA and those derived from the reaction between BSA and primary and secondary lipid oxidation products have shown antioxidant activity *in vitro* (*26*). On the other hand, the antioxidant activity of MP has been mainly attributed to the radical-scavenging and metal-chelating activities of certain peptides containing acidic side-chain amino acids (*27*). According to the present results, MP showed a more intense antioxidant activity in oil-in-water emulsions than BSA. Other authors have also found similar results when the antioxidant activity of BSA and several dairy proteins were compared (*12*). The different ability of BSA and MP to protect lipids against oxidation might be derived from the different amino acid composition and their three-dimensional structures. In this sense, MPs contain considerably high levels of amino acids with antioxidant potential, such as aromatic and sulfur-containing amino acids (*12, 24, 25*). However, the great antioxidant potential of MP should be mainly attributed to the activity of the most abundant amino acids in these proteins, namely, glutamic and aspartic acids (*28*). Saigas et al. (*27*) demonstrated that peptides containing those acidic amino acids display an antioxidant potential equivalent to that of α-tocopherol. The different three-dimensional structure of BSA and MP could also explain the results found in the present study because the lipid−protein interaction is greatly influenced by the structure of the proteins (*29*). In this sense, for a protein to act as a radical scavenger and metal chelator, the amino acid residues responsible for those activities should be surface-

exposed, and acidic amino acids from MPs fulfill this requirement (*24*). In addition, MPs display, unlike BSA, a complex three-dimensional structure that is responsible for their functional properties, including their emulsifying ability. When added to solutions of high ionic strength (around 0.6 M NaCl), MPs (mainly actomyosin) form soluble three-dimensional filaments (*30*). Under these conditions, actomyosin filaments exhibit a recognized ability to interact with lipids and form very stable emulsions that could enhance the oxidative stability of lipids by reducing, for instance, the interaction between lipid droplets and pro-oxidants from the aqueous phase.

The results obtained in the present study show that MPs were also more resistant to oxidative deterioration than BSA. According to oxidation essays described in previous papers (*12, 22*), the occurrence and extent of protein oxidation in the presence of oxidizing lipids largely depends upon the lipid−protein interactions during oxidative reactions and the susceptibility of the proteins, themselves, to be targets for oxidative deterioration. The loss of tryptophan natural fluorescence during storage of emulsions is a direct consequence of the oxidative degradation of this amino acid and is enhanced by the presence of lipid-derived radicals and hydroperoxides (*12, 22*). In fact, ROS derived from the first stages of lipid peroxidation, such as •OH, O_2•, and ROO•, can catalyze the abstract of a hydrogen from a susceptible amino acid residue, such as tryptophan, leading to the generation of a protein radical (*3*). BSA emulsions showed higher lipid oxidation rates than MP emulsions, and therefore, BSA was affected by the pro-oxidant effect of oxidizing lipids to a higher extent than MP. Moreover, MP and BSA might have different susceptibility to undergo oxidative reactions. Other authors previously reported large differences among different types of protein regarding their sensitivity to suffer oxidative deterioration. Measuring protein oxidation by means of tryptophan loss and carbonyl gain, Viljanen et al. (*12*) reported that lactalbumin is considerably more sensitive to oxidation than casein and BSA. The three-dimensional structure of the protein and its amino acid composition influence the susceptibility of proteins to suffer oxidative reactions and could explain the results obtained in the present study. As aforementioned, soluble MP form three-dimensional structures of considerable complexity. Actomyosin filaments show a tightly packed structure that is less accessible to oxygen and could hinder initiation of oxidation. Consistently, with tryptophan loss and protein carbonyl gain, BSA emulsions showed a more intense yellowish color than MP emulsions. Polymerized forms of Schiff bases that yield from the reaction between lipid-derived carbonyls and amino groups from proteins might be responsible for the yellowness development during the oxidation essay (*31*). Recently, Chelh et al. (*32*) reported that the detection of Schiff base fluorescence could be a liable procedure for protein oxidation measurement. In fact, Schiff bases formed during the oxidation essay in the present study might have contributed to the fluorescence measured at around 450 nm. It is plausible to consider that emulsions with higher lipid and protein oxidation rates (BSA emulsions) exhibited a more intense yellowish color as evidence of the formation of higher amounts of Schiff bases compared to MP emulsions.

Effect of Phenolics on Lipid and Protein Oxidation. The antioxidant potential of plant extracts or pure phenolic compounds is usually evaluated in model systems (liposomes, emulsions, and lipoproteins) before being used in food systems as inhibitors of lipid and protein oxidation (*12, 20*). The antioxidant activity of plant phenolics is mainly derived from their ability to act as radical scavengers and metal chelators (*33*). It is generally accepted that the effectiveness of plant phenolics as antioxidants is related to the stabilization of the phenoxyl radical by delocalization of an electron and the absence of suitable sites for O_2 attack, and it is enhanced by the presence of hydroxyl groups (*34*). However, the overall effect displayed by a phenolic compound in food and biological systems is affected by a variety of factors, including the composition and characteristics of the substrate, the stage and intensity of the oxidative reactions, and the localization of the phenolics (*35*). In the present study, the addition of the selected phenolic compounds resulted in a variety of effects, including both antioxidant and pro-oxidant effects. Three phenolic compounds, namely, cyanidin-3-glucoside, genistein, and gallic acid, showed a clear antioxidant effect, with that being more intense at higher concentrations. Anthocyanins have been profusely described as effective inhibitors of lipid oxidation in different lipid systems (*12, 17, 36*). Particularly, cyanidin-3-glucoside, a natural product from blackberry, has been found to exhibit a potent protective role against lipid and protein oxidation in liposomes (*12*) and oil-in-water emulsions (*17*). The aforementioned authors reported similar inhibition percentages to those found in the present study. The chemical structure of anthocyanins (flavylium cation) is responsible for its antioxidant potential because the 3′,4′-dihydroxy moieties in the B ring (catechol structure) provides reducing power, while the hydroxyl groups and the cation in A and C rings confer stability to the resulting radical (*37, 38*). In addition, Wang et al. (*38*) found that the 3-glycosylation in the C ring enhances the radical-scavenging activity of cyanidin-3-glucoside, which was similar to that exhibited by Trolox. The potent antioxidant activity of genistein was unexpected because the lack of the catechol structure in the B ring generally leads to limited scavenging activity (*39*). In isoflavones, the 3′-hydroxyl group in the B ring acts as a hydrogen donator, whereas the intact carbonyl group and the 5-hydroxyl group interact, contributing to the formation of a resonance-stabilized structure (*37*). Moreover, the 5-hydroxyl group in the A ring in conjunction with the 4-keto group from the C ring has been highlighted as a relevant metal-binding structure (*40*). Consistently, the intense antioxidant potential found for genistein in the present study should be attributed to concurrent radical-scavenging and metal-chelator activities, which hindered copper from acting as an initiator of the oxidative reactions. The three hydroxyl groups (pyrogallol structure) attached to the aromatic ring are responsible for the antioxidant activity of gallic acid (*37*). Gallic acid showed a more intense protective role against both lipid and protein oxidation than chlorogenic acid. These results disagree with the widespread thought that hydroxylated cinnamic acid derivatives are more efficient antioxidants than the benzoic acid counterparts (*41*). Chlorogenic acid was found to be an effective inhibitor of lipid oxidation in lipoproteins (*37*), while in the present study, the antioxidant effect against lipid oxidation was moderate (around 50% inhibition) and the antioxidant effect against protein oxidation was weak (between 10 and 20% inhibition). Above all phenolics tested in the present study, α-tocopherol exhibited the highest inhibitory effect against lipids and proteins. Previous studies in similar model systems found similar results (*12, 17*).

In opposition to the effect of the aforementioned compounds, (+)-epicatechin exhibited an antioxidant effect when added at low concentrations (50 μM), whereas it acted as a pro-oxidant at higher concentrations. Catechins are known to exhibit both antioxidant and pro-oxidant activities, and the overall effect depends upon the characteristics of the model system (*42*) and

the dose applied (*43*). In this sense, certain plant phenolics are known to lose their antioxidant activity at higher concentrations and behave as pro-oxidants by involvement in initiation reactions (*44*). Supporting the present results, Nakagawa et al. (*45*) have recently reported that copper ions can convert catechins from antioxidants to pro-oxidants through a mechanism not yet elucidated, and this interaction is dependent upon the concentration of catechin. In the above-mentioned study, catechins enhanced the formation of protein carbonyls from BSA, which is in good agreement with the present results. Among all phenolics tested, rutin was the only compound that showed a clear pro-oxidant effect, significantly promoting the formation of hexanal and protein carbonyls, the depletion of tryptophan residues, and the development of yellowness in the emulsions. Rutin is a glycoside derivative of quercetin, with recognized antioxidant activity, although its pro-oxidant potential is also well-documented (*39, 46*). Although the structural features for the pro-oxidant activity are not fully clear, Kessler et al. (*46*) considered that a hydroxyl group from rutin, such as the 7-hydroxyl moiety in the B ring, could be auto-oxidized and involved in initiation reactions, promoting the formation of superoxide radical and hydrogen peroxide. In agreement, Aboul-Enein et al. (*39*) found that rutin is able to promote the formation of hydrogen peroxide from the superoxide radical. Hydrogen peroxide could be decomposed into a hydroxyl radical in the presence of copper through the Fenton reaction and, therefore, enhance the oxidation of lipids and proteins.

In general, phenolic compounds showing a clear antioxidant activity (cyanidin-3-glucoside, genistein, and gallic acid) were more effective against lipid oxidation than in preventing protein oxidation, which is in agreement with previous studies (*12, 17*). The percent inhibition against hexanal formation displayed by these compounds at 200 μM was above 70%, whereas the percent inhibition against tryptophan loss and carbonyl gain was around 50 and 30%, respectively. These results suggest that phenolics could be mainly located in the inner layer of the interphase, exposed to the lipid phase, where lipid oxidation and hexanal formation takes place. By inhibiting lipid oxidation, phenolic compounds could have protected proteins from being oxidized in the inner layer of the interphase because primary lipid oxidation products (i.e., radicals and hydroperoxides) are known to initiate protein oxidation (*3*). However, the antioxidant effect that phenolics could directly exert on proteins was limited, and that could be explained by the likely occurrence of covalent binding between proteins and phenolic compounds that would hinder the antioxidant activity of the latter on proteins (*47*). Additionally, the protective role of cyanidin-3-glucoside, genistein, gallic acid, and α-tocopherol against tryptophan oxidation was clearly affected by the type of protein because the percent inhibition in BSA emulsions was 2-fold higher than in MP emulsions. These results suggest that protein structure and composition determine the number and characteristics of the binding sites, influence the availability and nature of protein−phenolic interactions, and hence, the overall effect of phenolics and α-tocopherol against protein oxidation. In this sense, certain plant phenolics, such as cyanidins, tend to have poor affinity for small globular proteins, such as BSA, whereas they bind preferentially to fibrous and structural proteins (*14*). In the present study, the interaction between certain phenolics and MPs could have decreased the ability of the former to act as radical scavengers. On the other hand, BSA has been shown to enhance the antioxidant activity of certain phenolics, such as catechins in oil-in water emulsions (*48*).

In conclusion, phenolic compounds protect MP and BSA against oxidation by scavenging lipid-derived radicals and by acting directly on proteins, although the overall effect depends upon the nature and consequences of the protein−phenolic interactions. The chemical structure of the phenolic compound as well as the nature and conformation of the protein are greatly influential. Further studies should shed light on the nature and specificity of protein−phenolic interactions and the effects on MP functionality and digestibility. The identification of efficient antioxidants is of great interest in food systems to maintain the shelf life and quality of the products. The effectiveness of phenolic compounds naturally present in fruits and vegetables (i.e., cyanidin-3-glucoside) as inhibitors of MP oxidation emphasize the benefit of using those as functional ingredients in muscle foods.

ACKNOWLEDGMENT

The authors acknowledge the technical support from Miikka Olin, Kirsti Risunen, and Maija Ylinen.

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Received for review June 11, 2008. Revised manuscript received September 21, 2008. Accepted October 2, 2008. Mario Estévez thanks the European Community for the economical support from the Marie Curie Intra-European Fellowship.

JF801784H