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Antioxidant Properties of Ferulic Acid and Its Related Compounds

HIROE KIKUZAKI,*,† MASASHI HISAMOTO,† KANAE HIROSE,† KAYO AKIYAMA,† AND HISAJI TANIGUCHI‡

Division of Food and Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585, Japan, and Industrial Technology Center of Wakayama Prefecture, 60 Ogura, Wakayama 649-6261, Japan

Antioxidant activity of 24 ferulic acid related compounds together with 6 gallic acid related compounds was evaluated using several different physical systems as well as their radical scavenging activity. The radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) decreased in the order caffeic acid > sinapic acid > ferulic acid > ferulic acid esters > *p*-coumaric acid. In bulk methyl linoleate, test hydroxycinnamic acids and ferulic acid esters showed antioxidant activity in parallel with their radical scavenging activity. In an ethanol−buffer solution of linoleic acid, the activity of test compounds was not always associated with their radical scavenging activity. Ferulic acid was most effective among the tested phenolic acids. Esterification of ferulic acid resulted in increasing activity. The activity of alkyl ferulates was somewhat influenced by the chain length of alcohol moiety. When the inhibitory effects of alkyl ferulates against oxidation of liposome induced by AAPH were tested, hexyl, octyl, and 2-ethyl-1-hexyl ferulates were more active than the other alkyl ferulates. Furthermore, lauryl gallate is most effective among the tested alkyl gallates. These results indicated that not only the radical scavenging activity of antioxidants, but also their affinity with lipid substrates, might be important factors in their activity.

KEYWORDS: Antioxidant; ferulic acid and esters; hydroxycinnamic acids; gallic acid and esters; radical scavenging activity; autoxidation; oil stability index; liposome

INTRODUCTION

Ferulic acid is one of the ubiquitous compounds in nature, especially rich as an ester form in rice bran pitch, which is yielded when rice oil is produced. Utilization of such industrial waste is important from the environmental point of view. It has been well-known that ferulic acid and γ-oryzanol, a mixture of monoesters consisting of ferulic acid and several kinds of triterpene alcohols as cycloartenol and 24-methylenecycloartanol, have an antioxidant activity (*1*−*3*). Such antioxidants are currently expected not only to prevent lipid oxidation in food but also to prevent free-radical-induced diseases such as cancer and atherosclerosis or aging caused by oxidative tissue degeneration (*4*). Therefore, there is a possibility of developing an application of rice bran pitch for human health.

In general, the inhibitory effect of antioxidants on lipid oxidation is influenced by the physicochemical state of the lipid substrate, and several evaluation systems using different physical conditions are required for better understanding of antioxidant properties (*5*). Although ferulic acid and its esters have been recognized as antioxidants, there are few reports on systematic evaluation of the antioxidant properties of ferulic acid and its derivatives in different conditions.

In this paper we describe the antioxidant activities of ferulic acid obtained from rice bran pitch and its synthetic esters, as well as some ferulic acid related compounds such as *p*-coumaric acid, caffeic acid, and sinapic acid in both bulk and multiphase systems. Gallic acid and its alkyl esters were also tested for their antioxidant activities in order to elucidate the influence of esterification on the antioxidant activity. Their antioxidant properties were evaluated on the basis of their inhibitory effects on the autoxidation of methyl linoleate in bulk system, on the oxidation of methyl linoleate under aeration and heating using the oil stability index (OSI) method, on the autoxidation of linoleic acid in an ethanol-buffer system, and on the oxidation of liposome induced by a water-soluble initiator, as well as their radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH).

MATERIALS AND METHODS

Materials. Ferulic acid, gallic acid, and their esters were supplied by Industrial Technology Center of Wakayama Prefecture, Japan. Caffeic acid, sinapic acid, *p*-coumaric acid, DPPH, α-tocopherol, L-ascorbic acid, butylated hydroxytoluene (BHT), 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), and *n*-octanol for determining

* To whom correspondence should be addressed (telephone +81-6-6605-2812; fax +81-6-6605-3086; e-mail kikuzaki@life.osaka-cu.ac.jp).
† Osaka City University.
‡ Industrial Technology Center of Wakayama Prefecture.

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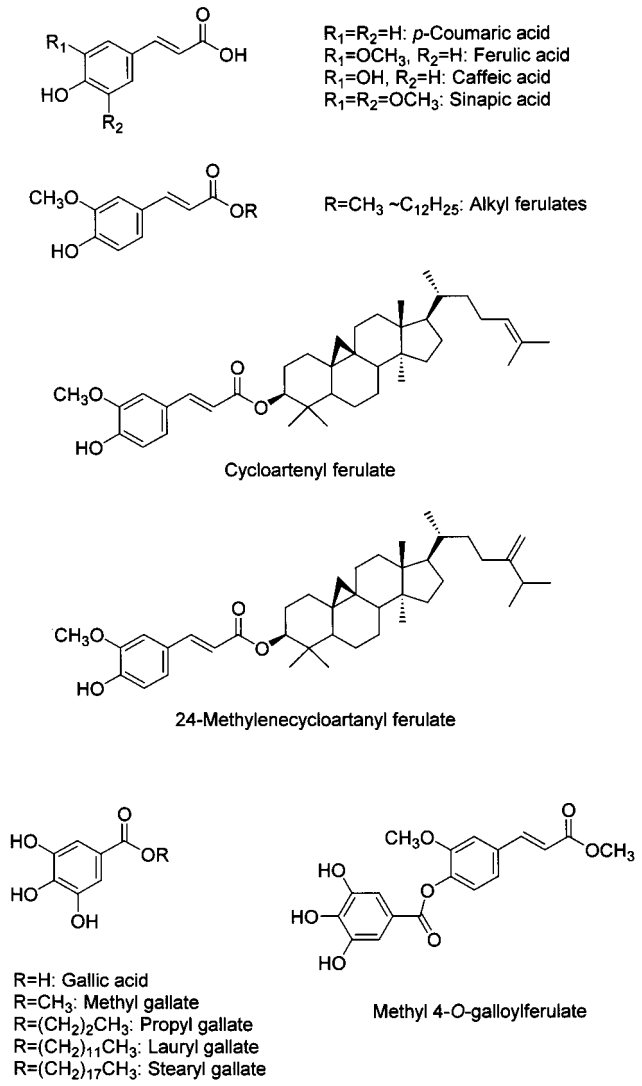

Figure 1. Structural formulas of ferulic acid related compounds and gallic acid related compounds.

partition coefficients were bought from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Linoleic acid and methyl linoleate were purchased from Tokyo Kasei Co. (Japan), and silicone oil TSF 451-100 was obtained from Toshiba Co. (Tokyo, Japan). Phosphatidylcholine (PC; 99% grade) from egg yolk and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). A 1.28 mM of PC hydroperoxides (PC-OOH) in chloroform/methanol (95:5, v/v) was kindly offered by Professor J. Terao (Tokushima University, Japan) and was stored in nitrogen at −20 °C. Linoleic acid was purified by distillation under reduced pressure. PC was purified by reversed-phase column chromatography according to the method of Terao et al. (*6*), just before the preparation of liposomes, to remove contaminant peroxides. All other chemicals used were of reagent grade. The structural formulas of test antioxidants are shown in **Figure 1**.

Evaluation of Scavenging Effect on DPPH Radicals. The compound to be tested was added into a 1 mM ethanol solution of DPPH (final concentration of 100 μM) to make final concentrations of 5, 10, 20, 40, and 80 μM. The mixture was shaken vigorously on a vortex mixer then incubated for 30 min in a water bath at 25 °C, after which the absorbance of the remaining DPPH was determined colorimetrically at 517 nm using a UV-2500PC UV−Vis spectrometer (Shimadzu, Kyoto, Japan). The scavenging activity of the tested compound was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution without test compounds (*7*, *8*). All analyses were carried out in triplicate.

Evaluation of the Inhibitory Effect on Autoxidation of Methyl Linoleate in Bulk Phase. To 1 g of methyl linoleate, 25 μL of the acetone solution of the test compound was mixed in a 20-mL vial (ϕ = 27, h = 55 mm) with a screw cap, and the mixture was shaken on a vortex mixer for 10 s. A 25-μL aliquot of acetone without sample was added for control. After purging the acetone with nitrogen, the mixture was placed in an oven at 40 °C in the dark. Final concentration of each sample was 0.05 μmol/g oil. An aliquot of oil sample was dissolved in ethanol, and the conjugated diene absorbance was measured at 234 nm with a UV-2500PC UV−Vis spectrometer every 24 h at 20 °C. Results were calculated as hydroperoxides in millimoles per kilogram (mM/kg) of oil based on an absorptivity of 26,000 for methyl linoleate hydroperoxides (*9*, *10*). All tests were run in triplicate.

Evaluation of the Antioxidant Activity by the OSI Method. The induction period for lipid oxidation under aeration and heating was measured with the Omnion oxidative stability instrument (Rockland, MA), which is equipped with an on-line computer that monitored the conductivity vs time and automatically plotted the inflection point or induction period in hours (OSI) (*11*). To the 5 g of the substrate oil, a mixture of 10% methyl linoleate, 90% silicone oil, and 100 μL of methanol solution of the test compound were mixed in a glass cylinder. Final concentration for gallic acid and its esters is 0.1 or 0.2 μmol/g oil and for the others is 0.4 μmol/g oil. The mixture was shaken on a vortex mixer for 30 s under nitrogen, and the reaction tube was connected to the conductivity measurement tube after preheating for 30 min without linking the conductivity measurement tube (*12*). Oxidation was carried out at 90 °C. All tests were run in triplicate.

Evaluation of the Antioxidant Activity in Ethanol-Buffer System. A mixture of 1 mL of a weighed test sample in 99.5% ethanol, 1 mL of 83.5 mM linoleic acid in 99.5% ethanol, 2 mL of 0.05 M phosphate buffer (pH 7.0), and 1 mL of water was placed in a vial (ϕ = 38, h = 75 mm) with a screw cap and then placed in an oven at 40 °C in the dark. For hydroxycinnamic acids and some ferulic acid esters, a ferric thiocyanate method was used. To 50 μL of the sample solution was added 4.85 mL of 75% ethanol and 50 μL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 50 μL of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of red color was measured at 500 nm every 24 h (*13*). For the evaluation of the antioxidant activity of a series of alkyl ferulates, the amounts of linoleic acid and produced hydroperoxides were measured by HPLC. The amounts of linoleic acid and its hydroperoxide were measured by the reversed-phase HPLC (Jasco PU-980, Tokyo, Japan), which was equipped with a multiwavelength detector (MD-910). Equal amounts (50 μL) of the sample solution and acetonitrile were mixed, and 2 μL of the mixture was injected into a Develosil ODS-HG-5 column (4.6 × 250 mm, Nomura Chemical Co., Ltd., Japan) with acetonitrile/water containing 0.1% phosphoric acid (90:10, v/v) at the flow rate of 1 mL/min. Linoleic acid (retention time 12.0 min) was monitored at 205 nm and its hydroperoxides (retention times 4.5 and 4.8 min) were monitored at 234 nm. All tests were run in triplicate.

Peroxidation of PC Liposomes Induced by Radical Generators. A purified PC in hexane and cholesterol in chloroform/methanol (95:5, v/v) were placed into a test tube, and after stirring with a vortex mixer, the mixture was evaporated under a stream of nitrogen and then in vacuo for 20 min to remove organic solvents. After 10 mM Tris−HCl buffer (pH 7.4) containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) was added to the lipid film in a test tube, the suspension was mixed with a vortex mixer for 1 min followed by ultrasonication for 1 min to prepare multilamellar vesicles (MLV). The suspension of MLV was extruded 21 times through a polycarbonate filter (100-nm pore diameter) mounted in an extrusion apparatus (Lipsofast, Avestn Inc., Ottawa, ON) to make large unilamellar vesicles (LUV). To a test tube with screw cap were added 500 μL of the LUV suspension and 350 μL of Tris−HCl buffer and 50 μL of the 1-mM ethanolic sample solution. After mixing, the mixture was preincubated at 37 °C for 10 min. The peroxidation was started by the addition of 100 μL of 200 mM AAPH solution, and the reaction mixture was incubated at 37 °C in the dark with continuous shaking. The final concentrations in the incubation mixture were as follows: PC 5 mM, cholesterol 2.5 mM, test samples 50 μM, and AAPH 20 mM (*14*). At regular intervals, 25 μL of each reaction mixture was withdrawn and put into 75 μL of methanol. Instantly, an aliquot of the mixture was injected into a HPLC column (TSK-gel Octyl 80Ts, 4.6 × 150 mm, TOSOH, Tokyo, Japan),

Table 1. Radical Scavenging and Antioxidant Activities, and Partition Coefficients in *n*-Octanol/PBS System of Ferulic Acid Related Compounds

compound	DPPH radical scavenging activity (%)[a]	OSI[b] (0.4 μmol/g oil)	antioxidant index[c]	partition coefficient[d]
control	0	1	1	–
p-coumaric acid	7.0 ± 0.8 g	2.37 ± 0.06 cd	–	0.22 ± 0.02 b
ferulic acid	27.3 ± 0.8 d	2.97 ± 0.11 cd	4.2 ± 1.1 ab	0.13 ± 0.01 a
caffeic acid	49.6 ± 0.6 a	17.64 ± 3.50 a	–	0.08 ± 0.01 a
sinapic acid	33.2 ± 0.4 c	11.79 ± 1.74 b	–	0.09 ± 0.01 a
methyl ferulate	18.1 ± 1.0 f	3.23 ± 0.14 cd	4.7 ± 1.1 a	0.95 ± 0.03 c
ethyl ferulate	21.0 ± 0.5 f	3.56 ± 0.12 cd	4.9 ± 0.5 a	0.96 ± 0.03 c
propyl ferulate	22.3 ± 0.6 f	3.52 ± 0.33 cd	4.5 ± 0.8 ab	–
butyl ferulate	20.3 ± 1.4 f	3.34 ± 0.02 cd	4.7 ± 1.3 a	0.95 ± 0.02 c
pentyl ferulate	20.2 ± 0.6 f	3.26 ± 0.18 cd	5.5 ± 1.1 a	–
hexyl ferulate	20.1 ± 0.4 f	3.04 ± 0.06 cd	5.8 ± 0.4 a	0.97 ± 0.01 c
heptyl ferulate	21.4 ± 0.9 f	2.94 ± 0.02 cd	5.6 ± 1.6 a	–
octyl ferulate	23.0 ± 0.8 f	2.92 ± 0.07 cd	4.8 ± 1.0 a	0.97 ± 0.01 c
nonyl ferulate	21.1 ± 0.3 f	3.05 ± 0.06 cd	5.5 ± 0.9 a	–
decyl ferulate	20.3 ± 0.2 f	2.96 ± 0.18 cd	4.7 ± 0.8 a	0.98 ± 0.01 c
undecyl ferulate	19.1 ± 1.3 f	3.01 ± 0.14 cd	4.1 ± 0.2 ab	–
dodecyl ferulate	20.4 ± 0.3 f	2.85 ± 0.17 cd	5.2 ± 0.7 a	0.97 ± 0.01 c
isopropyl ferulate	20.7 ± 0.4 f	3.55 ± 0.03 cd	–	–
t-butyl ferulate	21.1 ± 0.1 f	3.35 ± 0.04 cd	–	–
2-methyl-1-butyl ferulate	19.8 ± 1.3 f	2.88 ± 0.03 cd	–	–
2-ethyl-1-hexyl ferulate	19.3 ± 0.1 f	3.17 ± 0.06 cd	–	0.98 ± 0.01 c
3,5,5-trimethyl-1-hexyl ferulate	26.2 ± 1.2 e	2.90 ± 0.06 cd	–	–
cycloartenyl ferulate	21.8 ± 0.4 ef	2.92 ± 0.13 cd	–	0.99 ± 0.01 c
24-methylenecycloartanyl ferulate	20.4 ± 1.2 f	2.25 ± 0.07 d	–	0.99 ± 0.02 c
γ-oryzanol[e]	21.2 ± 1.5 f	2.98 ± 0.26 cd	–	–
α-tocopherol	41.8 ± 1.0 b	9.21 ± 0.46 bc	1.2 ± 0.2 b	–
BHT	29.2 ± 1.1 cd	6.68 ± 0.24 c	5.2 ± 1.6 a	–

[a] The concentration of DPPH ethanolic solution was 100 μM and the concentration of compounds was 20 μM. [b] OSI is defined as the induction time of lipid oxidation at 90 °C with antioxidant over the induction time of control with no additive (control). [c] Antioxidant index is expressed as the time at which linoleic acid is 50% oxidized with antioxidant divided by that with no additive (control). [d] Partition coefficient is expressed as the amount of test compound dissolved in *n*-octanol divided by the amount (200 μM) added to the *n*-octanol/PBS solution. [e] The molecular weight of γ-oryzanol was estimated at 602, a molecular weight of cycloartenyl ferulate which is a major component of γ-oryzanol. Each value is the mean of triplicate measurements, and SD means standard deviation of measurement. Values within a column with different letters are significantly different at $P < 0.05$.

eluted with methanol/water (95:5, v/v) at the flow rate of 1 mL/min with the wavelength of 235 nm for detection. The standard PC-OOH solutions were prepared at concentrations in the range of 0.0128 to 0.128 mM in the dark at 4 °C and were immediately analyzed by HPLC. The amount of PC-OOH in each tested sample was estimated with the calibration curve (the r^2 value was 0.997) prepared with standard PC-OOH solutions (*15*). All tests were run in triplicate and averaged.

Measurement of Partition Coefficient in Octanol/PBS. A solution (200 μM) of the test compound in *n*-octanol was kept at 40 °C for 1 h and the absorbance value at 254 nm was measured (A_0). An equal volume of phosphate-buffered saline (PBS, pH 7.4) was added to the organic solution, and the mixture was stirred using a vortex mixer for 1 min, followed by keeping at 40 °C for 1 h. After the mixture was centrifuged at 1000 rpm for 10 min, the absorbance at 254 nm of the organic layer was measured (A_x). The partition coefficient was expressed as A_x/A_0. A solution of *n*-octanol saturated with water was used as a blank. All tests were run in triplicate.

Statistical Analysis. One-way analysis of variance (ANOVA) based on data collected from duplicate or triplicate determinations was carried out. Significance was established at $p < 0.05$.

RESULTS

Scavenging Effect on DPPH Radicals. It is generally accepted that the electron-donating ability of chemical substances results in their antioxidant activity toward lipid oxidation. DPPH radical-scavenging test is one of the short methods for investigation of the hydrogen donating potency (*7*).

Scavenging ability of all test compounds increased with concentration in the range of 1 to 100 μM (data not shown). **Table 1** shows the scavenging effects of ferulic acid related compounds on DPPH radical at a concentration of 20 μM. All ferulates scavenged about 20% DPPH radical and there were few significant differences among them. The activity decreased in the order caffeic acid > α-tocopherol > sinapic acid = BHT = ferulic acid > ferulates > *p*-coumaric acid. In the case of gallic acid and alkyl gallates, they scavenged about 80% DPPH radical and there were slight significant differences among them (**Table 2**).

Table 2. Radical Scavenging and Antioxidant Activities, and Partition Coefficients in *n*-Octanol/PBS System of Gallic Acid Related Compounds

compound	DPPH radical scavenging activity (%)[a]	OSI[b] (0.1 μmol/g oil)	OSI (0.2 μmol/g oil)	partition coefficient[c]
control	0	1	1	–
gallic acid	75.7 ± 1.1d	7.18 ± 1.87 ab	9.44 ± 0.93 b	0.02 ± 0.02 a
methyl gallate	86.3 ± 0.5 a	9.65 ± 2.75 a	27.16 ± 1.09 a	0.79 ± 0.01 b
propyl gallate	87.1 ± 2.3 ab	6.80 ± 0.24 ab	11.92 ± 0.02 b	0.95 ± 0.01 c
lauryl gallate	81.6 ± 2.2 abc	8.78 ± 0.68 ab	11.45 ± 0.92 b	0.95 ± 0.01 c
stearyl gallate	79.0 ± 1.7 cd	8.11 ± 0.84 ab	9.08 ± 1.13 b	0.95 ± 0.01 c
α-tocopherol	–	4.99 ± 1.11 ab	5.83 ± 0.88 c	–
BHT	–	4.41 ± 0.50 b	4.45 ± 0.24 c	–

[a–c] See **Table 1**. Each value is the mean of triplicate measurements, and SD means standard deviation of measurement. Values within a column with different letters are significantly different at $P < 0.05$.

Inhibitory Effect on Autoxidation of Bulk Methyl Linoleate. Formation of hydroperoxides in bulk methyl linoleate at 40 °C was estimated based on the absorbance due to a conjugated methyl linoleate yielded during the initial oxidation stage. Tested hydroxycinnamic acids except for *p*-coumaric acid showed inhibitory effects on hydroperoxide formation. Especially caffeic acid suppressed hydroxyperoxide accumulation

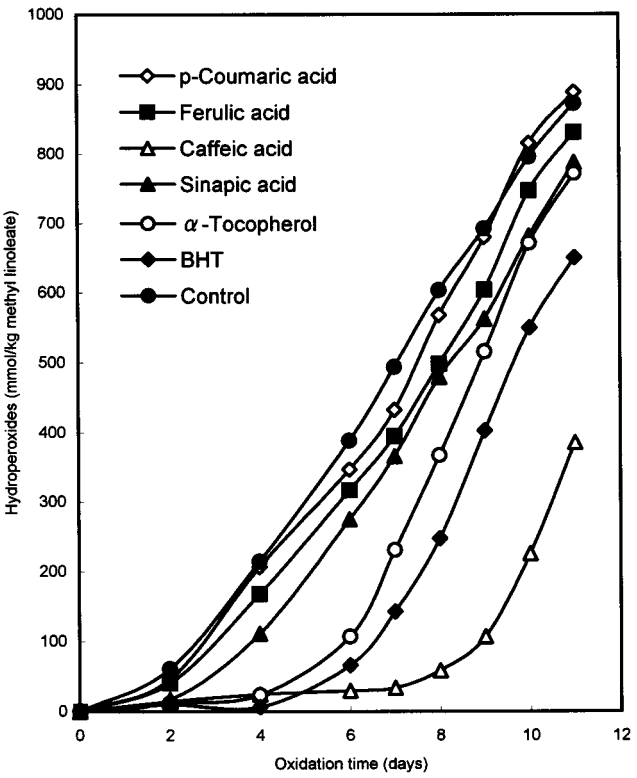

Figure 2. Inhibition of the formation of hydroperoxides in bulk methyl linoleate by hydroxycinnamic acids. The concentration of compounds was 0.05 μmol/g methyl linoleate. The oxidation was carried out at 40 °C.

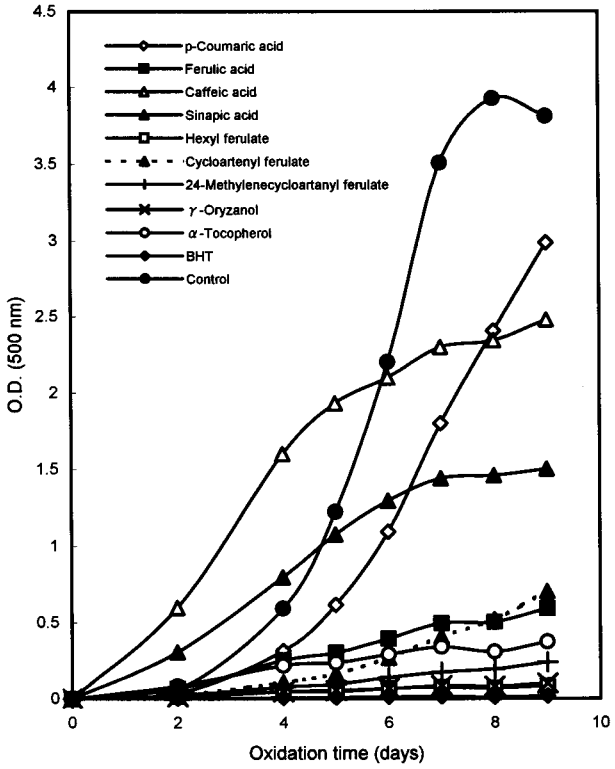

Figure 3. Inhibition of the formation of hydroperoxides in ethanol-buffer solution of linoleic acid (pH 7.0) by ferulic acid related compounds. The concentration of compounds was 200 μM. The oxidation was carried out at 40 °C. Formation of hydroperoxides was monitored by the ferric thiocyanate method.

with a clear induction period. The activity decreased in the order caffeic acid > BHT > α-tocopherol > sinapic acid > ferulic acid > *p*-coumaric acid (**Figure 2**). All alkyl ferulates were less active than ferulic acid and the inhibition was not significantly different among alkyl ferulates (data not shown). In the case of gallic acid and alkyl gallates, gallic acid had a tendency to be more active than its alkyl esters though there was no significant difference between them. Within alkyl gallates, methyl gallate showed slightly higher inhibition than the other gallates (data not shown).

Evaluation of the Antioxidant Activity Using OSI Method. The OSI method is commonly accepted as one of the evaluation methods for the stability of fats and oils (*11*). In this method, oil stability is evaluated by measuring the conductivity due to volatile organic acids evolved during the oxidation of fats and oils. Recently, we established the procedure of measurement of antioxidant activity by the OSI method using the model oil system with methyl linoleate−silicone oil as substrate oil (*12*).

As shown in **Table 1**, all test compounds extended the induction time of the model substrate oil at 90 °C. Comparing the activity of hydroxycinnamic acids at the concentration of 0.4 μmol/g oil, the activity decreased in the order caffeic acid > sinapic acid ≅ α-tocopherol ≅ BHT ≅ ferulic acid ≅ *p*-coumaric acid. As to ferulic acid esters, they showed almost the same activity as ferulic acid, prolonging the induction time 2 to 3 times. In the case of gallic acid and its alkyl esters, the activity decreased methyl gallate > propyl gallate ≅ lauryl gallate ≅ gallic acid ≅ stearyl gallate > α-tocopherol ≅ BHT at the concentration of 0.2 μmol/g oil (**Table 2**).

Evaluation of the Antioxidant Activity in Ethanol−Buffer System. Because real food generally consists of multiple phases in which lipid and water coexist with some emulsifier, instead of in the bulk phase, an antioxidant assay using heterogeneous system such as oil-in-water emulsion was also required. Autoxidation of linoleic acid in ethanol−buffer system is one of the simple conditions of oxidation in heterogeneous system for evaluation of effects of antioxidants (*16*).

As shown in **Figure 3**, *p*-coumaric acid slightly suppressed formation of hydroperoxide, whereas caffeic acid and sinapic acid showed prooxidative behavior at the first stage of autoxidation at the concentration of 200 μM. At the same concentration, ferulic acid was the most effective within test hydroxycinnamic acids and the activity was almost the same as that of α-tocopherol. Ferulic acid esters such as hexyl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and γ-oryzanol inhibited hydroperoxide formation more strongly than ferulic acid. Especially hexyl ferulate and γ-oryzanol showed the strong activity comparable to BHT. To compare the activity among alkyl ferulates, both formation of hydroperoxide and decrease of linoleic acid were measured by HPLC. At the point when hydroperoxide formation accelerated, a rapid decrease of linoleic acid was observed. The activity of each alkyl ferulate was expressed as the antioxidant index, the ratio of the length of time (days) at which linoleic acid is 50% oxidized to that without any additive (control). When the activity of hexyl ferulate was measured at the concentrations 12.5, 25, 50, and 100 μM, the activity increased with an increase in the concentration in the range tested (data not shown). The activity of alkyl ferulates was expressed as the antioxidant index at the concentration of 50 μM (**Table 1**). At this concentration, alkyl ferulates showed the antioxidant index of 4.1−5.8. Pentyl, hexyl, and heptyl ferulates seemed to be more active than the other ferulates.

Evaluation of the Antioxidant Activity in Liposomes. When liposome oxidation was induced by a water-soluble radical generator of AAPH, most test samples suppressed the

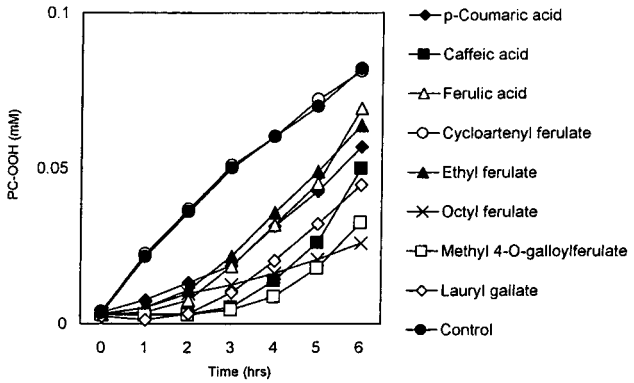

Figure 4. Effect of ferulic acid related compounds and gallic acid related compounds on the AAPH-induced peroxidation of egg yolk PC liposomes. The reaction mixture consisted of egg yolk PC (5 mM), AAPH (20 mM), and antioxidant (50 μM) in 10 mM Tris-HCl (pH 7.4) containing 0.5 mM DTPA.

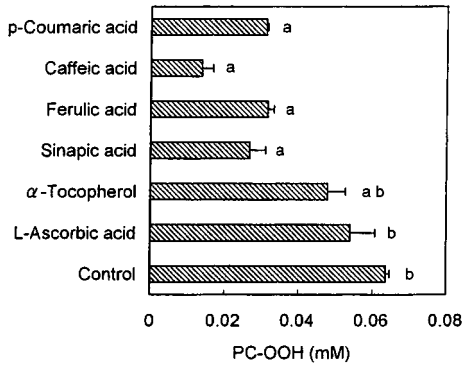

Figure 5. Inhibition of the AAPH-induced peroxidation of egg yolk PC liposomes by hydroxycinnamic acids. Each column expresses the mean of the amounts of PC-OOH after 4 h incubation based on triplicate measurements. Values with different letters are significantly different at $p < 0.05$.

accumulation of PC-OOH with a clear induction period, except cycloaltenyl ferulate as shown in **Figure 4**. All test hydroxycinnamic acids significantly inhibited formation of PC-OOH, and there was a tendency that caffeic acid was most effective among them after a 4-h incubation (**Figure 5**). Test ferulic acid esters except cycloartenyl and 24-methylcycloartanyl ferulates showed a significant activity (**Figure 6**). Comparing the effects of ferulic acid and its esters, hexyl, octyl, and 2-ethyl-1-hexyl ferulates were significantly more active than ferulic acid. In the inhibitory effects of the ferulate series, higher or lower carbon chain homologues than hexyl and octyl ferulates were less active. In the case of gallic acid and gallates, they significantly suppressed PC-OOH formation, and alkyl gallates were more active than gallic acid. Especially, lauryl gallate was most active among the alkyl gallate series. In addition, we also measured the activity of methyl 4-*O*-galloylferulate, which showed the highest activity among gallic acid related compounds, indicating high radical scavenging activity (92.7% at 20 μM) and OSI (15.8 at the concentration of 0.4 μmol/g oil, 90 °C) (**Figure 7**).

Partition Coefficient in Octanol/PBS. Partition coefficient based on the amount of each test compound dissolved in *n*-octanol divided by the amount added to the *n*-octanol/PBS solution was determined to estimate its lipophilicity. The partition coefficients of hydroxycinnamic acids were in the range of 0.09 to 0.22, indicating their low lipophilicity. The partition coefficients of alkyl ferulates were in the range of 0.95 to 0.98, whereas that of ferulic acid was 0.13 (**Table 1**). **Table 2** shows

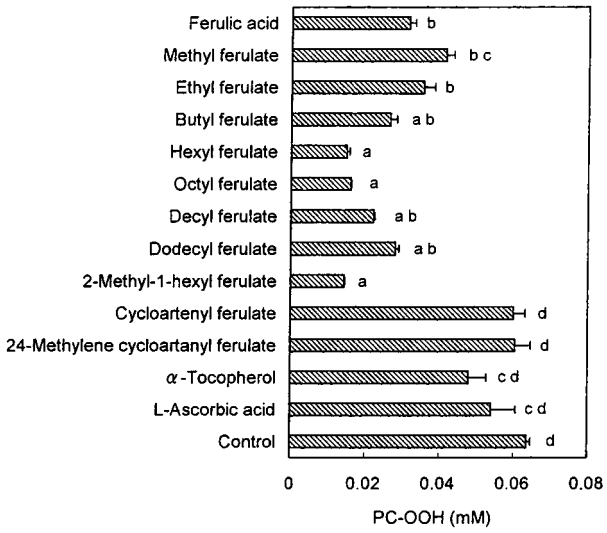

Figure 6. Inhibition of the AAPH-induced peroxidation of egg yolk PC liposomes by ferulic acid and its esters. Each column expresses the mean of the amounts of PC-OOH after 4 h incubation based on triplicate measurements. Values with different letters are significantly different at $p < 0.05$.

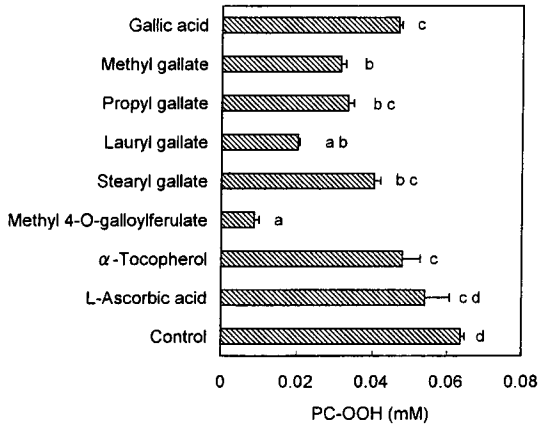

Figure 7. Inhibition of the AAPH-induced peroxidation of egg yolk PC liposomes by gallic acid related compounds. Each column expresses the mean of the amounts of PC–OOH after 4 h incubation based on triplicate measurements. Values with different letters are significantly different at $p < 0.05$.

the partition coefficients of gallic acid related compounds. Gallic acid showed a very low value of 0.02. Methyl gallate showed 0.79 of coefficient, significantly lower than those of the other alkyl gallates (0.95). In addition, methyl 4-*O*-galloylferulate had the highest coefficient of 0.97 (data not shown).

DISCUSSION

It is proposed nowadays that the use of more than one condition of oxidation is required to evaluate antioxidants (*5*). Although much attention has been paid to the potential activity of hydroxycinnamic acids and their related compounds against lipid oxidation for practical use as natural antioxidants on account of their widespread occurrence in plants (*17*−*19*), further systematic information on their antioxidant properties in different conditions needs to be investigated. The present study demonstrates the antioxidant properties of hydroxycinnamic acids and ferulic acid esters, as well as gallic acid and alkyl gallates, in both bulk and multiphase systems together with their radical scavenging activities.

The scavenging ability of hydroxycinnamic acids against DPPH radical was dependent on the number of hydroxyl groups on the benzene ring and *ortho* substitution with the electron donor methoxy group which increases the stability of the phenoxy radical as expected from the results of Chen and Ho (*20*) and Pekkarinen et al. (*21*). The scavenging activity order was consistent with the antioxidant activity order against autoxidation of bulk methyl linoleate at 40 °C, suggesting that their antioxidant efficiencies result in their electron-donating ability. The results were in accordance with those reported by Pekkarinen et al. who measured the inhibition effects of some phenolic acids, including hydroxycinnamic acids, on the formation of hydroperoxides in bulk methyl linoleate at 40 °C (*21*). Furthermore, when measured by the OSI method at 90 °C in this study, the activity order of hydroxycinnamic acids was in good agreement with that in the case of autoxidation of the bulk methyl linoleate at 40 °C. These results suggested that the change in temperature did not affect their antioxidant activity order. Marinova and Yanishlieva (*22*) evaluated the antioxidant efficiency of ferulic acid and α-tocopherol on lard oxidation at 25, 50, 75, and 100 °C and reported that α-tocopherol showed behavior different from that of ferulic acid. α-Tocopherol was more effective at extending the induction period and reducing the rate of autoxidation as the temperature was increased. They suggested that the participation of α-tocopherol in the initiation or propagation in lipid oxidation changed with rising temperature. We obtained the same results that α-tocopherol was more effective at a higher temperature than at a lower temperature. On the contrary, their results indicated that increasing the temperature did not influence the effect of ferulic acid on the induction period or the rate of autoxidation, leading to the conclusion that the mechanism of antioxidant action of ferulic acid was the inhibition of initiation of radical reaction in oxidation process regardless of temperature. Cuvelier et al. evaluated the antioxidant activity of some phenolic acids based on measuring the disappearance of methyl linoleate in dodecane under heating (110 °C). They reported that the activity order was caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid, and the activity depended on their stability of the aryloxy radicals (*17*). Marinova and Yanishlieva also obtained the same activity order by determination of hydroperoxides, the primary oxidative products at 100 °C (*18*). Huang et al. proposed that both the formation of hydroxyperoxides and the decomposition of hydroperoxides needed to be measured to clarify the mechanism of antioxidant action (*23*). In the OSI method, formation of volatile organic acids derived from hydroperoxides during the oxidation process was evaluated. Our results indicated that the activity order of hydroxycinnamic acids obtained from the OSI test was compatible with those reported by Cuvelier et al. and Marinova and Yanishlieva. It is suggested that within the group of hydroxycinnamic acids, they might act with the same efficacy toward the different stages of oxidation, and their antioxidant ability in bulk oil at any different temperatures could be extrapolated to their radical scavenging activity.

In this study, test alkyl ferulates showed almost the same scavenging activity against DPPH radical and their activity was lower than that of ferulic acid. This finding reflected the results of their antioxidant activity in bulk methyl linoleate at 40 °C. Ferulic acid suppressed formation of hydroperoxide more than alkyl ferulates. However, in high-temperature oxidation, the activity of ferulic acid and its esters were almost the same (**Table 1**). In the case of gallic acid and the corresponding alkyl esters, gallic acid showed stronger activity than its esters at 40 °C. Furthermore, methyl gallate had a tendency to possess a higher activity within the group of alkyl gallates, and based on the OSI method at 90 °C, methyl gallate showed a significant activity compared to that of the other gallates (**Table 2**). These results suggested that, concerning gallic acid and alkyl gallates, the affinities of antioxidants toward the air−oil interfaces in bulk oil might participate in their activity as well as their radical scavenging ability. The partition coefficients of antioxidants in the *n*-octanol/PBS system could be explained by their affinity with lipid in some cases. It seemed that the higher polarity of methyl gallate within the group of tested alkyl gallates might result in higher effectiveness on oxidation of bulk methyl linoleate in good agreement with the "polar paradox", according to which polar antioxidants are more efficient in an apolar medium, and conversely, apolar antioxidants are more efficient in a polar medium (*24*). In addition, the fact that gallic acid, having 0.03 of the partition coefficient, showed the strongest activity at 40 °C supports this "polar paradox". The higher effectiveness of ferulic acid than its esters at 40 °C also might be influenced by the difference in their partition coefficients, as well as their radical scavenging abilities. In the case of hydroxycinnamic acid, the difference of their radical scavenging activities might govern their antioxidant activity in bulk oil more than the difference of their affinities with lipid.

The results obtained from the antioxidant assay using the ethanol−buffer system differed from those of the radical scavenging and the antioxidant activities in the bulk phase. Ferulic acid showed the strongest action among test hydroxycinnamic acids, whereas caffeic acid and sinapic acid rather slightly promoted hydroperoxide formation at the initial stage of autoxidation of linoleic acid. This phenomenon was also observed in gallic acid (data not shown). In this case, the radical scavenging ability did not reflect the antioxidant activity. Pekkarinen et al. reported that caffeic acid slightly promoted hydroperoxide formation in emulsified methyl linoleate, but methoxylated phenolic acids, such as ferulic acid and sinapic acid, acted as antioxidants (*21*). Prooxidant behavior of hydrophilic compounds, such as phenolic acids in heterophasic systems, was observed in tea catechins (*25*). The antioxidant activity in multiphase systems might be affected by several parameters, including concentration of antioxidants, partitioning, and interactions with other compounds such as contaminating transition metals. Further investigation should be conducted to elucidate the antioxidant mechanism in heterophasic system. Esters of ferulic acid were more active than ferulic acid in the ethanol−buffer system. Furthermore, the activity was appreciably dependent on their alkyl chain length and the optimum chain lengths of alcohol moieties were from five to eight carbons within ferulates. This result suggested that affinity of ferulates to substrate oil might have some influence on their activity in this system.

A liposomal system has been used as a model of phospholipid bilayers constituting cellular and subcellular membranes to predict antioxidant abilities toward biological cell membranes. In addition, localization of antioxidants based on their hydrophilic or hydrophobic properties in the phospholipid bilayers resulting from the regular and tight steric structure of bilayers is available for elucidation of antioxidant mechanism. The order of activity toward liposome oxidation induced by AAPH was slightly reflected by the radical scavenging ability within hydroxycinnamic acids. In the case of alkyl ferulates, the inhibitory effects were influenced by their alkyl chain lengths of the alcohol moiety, although their radical scavenging potency was almost the same. Hexyl, octyl, and 2-ethyl-1-hexyl ferulates

were appreciably more active than the other alkyl ferulates which have a carbon chain length shorter or longer than that of the hexyl or octyl ferulates. Concerning gallic acid and its alkyl esters, lauryl gallate was most effective among the tested alkyl gallates, although their radical scavenging abilities were almost the same. It is recognized that peroxyl radicals produced by AAPH in the aqueous phase attack phospholipids at the membrane surface (*26*), suggesting that antioxidants need to be located near the surface of the membrane in order to act. Nakayama et al. reported that affinity of antioxidants for lipid bilayers governed their effectiveness on liposome oxidation (*27*). They measured the amounts of gallic acid, methyl gallate, propyl gallate, and lauryl gallate incorporated into the lipid bilayers of liposome. They referred to the affinity factor, which was calculated by amount of the compound incorporated into the liposomes divided by the amount added to the solution. Their results indicated that gallic acid was not incorporated into liposome at all, and that the incorporation percentages of alkyl gallates were 1% of methyl gallate, 10% of propyl gallate, and 84% of lauryl gallate. Taking into consideration these results, it might be concluded that lauryl gallate had the highest affinity toward liposome bilayers among the alkyl gallates tested in our study, because stearyl gallate was somewhat less active than lauryl gallate. The fact that methyl 4-*O*-galloylferulate showed the highest activity suggested that this gallate had a significant chain length. However, more information on the activity of alkyl gallates with different chain lengths is necessary in order to determine the optimum chain length. The effective chain length discrepancy between ferulates and gallates might arise from the difference in polarities of their acidic parts. Higher polarity of a phenoxyl group in alkyl gallates than that in alkyl ferulates might require a somewhat longer alkyl chain in the alcohol part. As mentioned above, although the activity order of hydroxycinnamic acids was in accordance with their radical scavenging abilities, there was no significant difference among them. Hashimoto et al. indicated that the affinities of tea catechins for the lipid bilayers corresponded to their partition coefficients (*28*). It is appeared that participation of both the radical scavenging ability of hydroxycinnamic acids and their affinity for the surface of phospholipids might result in their antioxidant activity toward liposome oxidation. In the case of cycloartenyl and 24-methylenecycloartanyl ferulates, they showed no activity in the liposomal system, although both showed almost the same radical scavenging activities and partition coefficients (both 0.99) as the tested alkyl ferulates. This suggests that their bulky structures might hinder their contact with lipid bilayers in liposomes.

In this study, the antioxidant properties of ferulic acid and its related compounds were investigated. The antioxidant activities of ferulic acid and its esters, as well as those of the other tested hydroxycinnamic acids, were in good agreement with their radical scavenging activities when measured using a bulk oil system at 40 °C and 90 °C. Ferulic acid showed the most effective antioxidant activity among the tested hydroxycinnamic acids against autoxidation of linoleic acid in the ethanol−buffer system. Esterification of ferulic acid increased the activity. In the liposome system, hexyl, octyl, and 2-ethyl-1-hexylferulates showed the strongest activities among the tested ferulic acid and its esters, whereas cycloartenyl and 24-methylenecycloartanyl ferulates showed no activity. It seemed that in addition to the radical scavenging activity of an antioxidant, both its polarity and three-dimensional interaction with lipid bilayers might participate in the exertion of the antioxidant activity toward liposome oxidation.

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