Molecular Design of Antibrowning Agents†

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Tyrosinase inhibitory and antioxidant activity of gallic acid and its series of alkyl chain esters were investigated. All inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by mushroom tyrosinase. However, gallic acid and its short alkyl chain esters were oxidized as substrates yielding the colored oxidation products. In contrast, the long alkyl chain esters inhibited the enzyme activity without being oxidized. This indicates that the carbon chain length is associated with their tyrosinase inhibitory activity, presumably by interacting with the hydrophobic protein pocket in the enzyme. On the other hand, the esters, regardless their carbon chain length, showed potent scavenging activity on the autoxidation of linoleic acid and 1,1-diphenyl-2-*p*-picryhydrazyl (DPPH) radical, suggesting that the alkyl chain length is not related to the activity. The effects of side-chain length of gallates in relation to their antibrowning activity are studied.

Keywords: *Tyrosinase inhibitors; antioxidants; antibrowning agents; molecular design; gallic acid; dodecyl gallate*

INTRODUCTION

In most foods, the browning process has two components: enzymatic and nonenzymatic oxidation. This unfavorable darkening from oxidation generally results in a loss of nutritional and market values (Friedman, 1996). The enzymatic oxidation can be prevented by tyrosinase (EC 1.14.18.1) inhibitors, and the nonenzymatic oxidation can be protected by antioxidants. Tyrosinase, also known as polyphenol oxidase (PPO) (Zawistowski et al., 1991; Whitaker, 1995; Mayer and Harel, 1998), is widely distributed in microorganisms, animals, and plants. Plant PPO contributes negatively to the color quality of plant-derived foods and beverages. This unfavorable darkening from enzymatic oxidation has been of some concern. It is responsible for not only browning in plants but also melanization in animals. For example, the unfavorable browning caused by tyrosinase on the surface of seafood products has also been of great concern (Ogawa et al., 1984). In addition, tyrosinase inhibitors have become increasingly important in medicinal (Mosher et al., 1983) and cosmetic (Maeda and Fukuda, 1991) products in relation to hyperpigmentation. Hence, tyrosinase inhibitors should have broad applications.

Although a large number of naturally occurring tyrosinase inhibitors have already been described (Pifferi et al., 1974; Mayer and Harel, 1979; Passi and Nazzaro-Porro, 1981; Mayer, 1987), their individual activity is usually not potent enough to be considered for a practical use. A possible solution to cross this hurdle is combining two or more inhibitors to increase the total biological activity or their synthetic modification. The emphasis in this paper was placed on an example for the latter case. It should be noted however that safety is a primary consideration for food additives since they can be utilized in unregulated quantities. In our continuing search for mushroom tyrosinase inhibitors from plants (Kubo, 1997), gallic acid was isolated from several plants (Kubo and Matsumoto, 1985; Matsuo et al., 1997) and identified as a potential PPO inhibitor. In addition, gallic acid is a frequent constituent of hydrolyzable tannins in many plants. On the other hand, the three gallates—propyl, octyl, and dodecyl—are currently permitted for use as antioxidant additives in food (Aruoma et al., 1993); therefore, a series of its alkyl chain esters was synthesized and their effects on mushroom tyrosinase were investigated to determine if the gallates also inhibit this enzyme. The aim of this research is to design multifunctional antibrowning agents for foods.

MATERIALS AND METHODS

General Methods. All the procedures used were the same as previously described (Kubo and Kinst-Hori, 1998; Haraguchi et al., 1997). UV−visible spectra were recorded by a Hitachi 100-80 spectrophotometer.

Chemicals. Gallic acid and its methyl ester, L-DOPA, pyrogallol, and DPPH were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. Xanthine oxidase, L-tyrosine, propyl, ethyl, and dodecyl gallates, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO).

Synthesis. To a solution of gallic acid (2 mM) and the corresponding alcohol (2 mM) in THF (6 mL) cooled at 0 °C was added a solution of dicyclohexylcarbodiimide (DCC) (4.2 mM) in THF (6 mL). After the solution had been allowed to

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stir for 20 h, the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate several times and filtered. The filtrate was washed successively with dilute aqueous citric acid solution, saturated aqueous sodium hydrogen carbonate solution, and water, dried ($MgSO_4$), and evaporated. The crude products were purified by chromatography (SiO_2; elution with chloroform−methanol, 98:2). Structures of the synthesized esters were established by spectroscopic methods (UV, IR, MS, and NMR). The best yield (87%) was obtained with octyl gallate. It should be pointed out that synthesis was achieved up to eicosanyl (C_{20}) gallate but the assay data were obtained unequivocally only up to dodecyl (C_{12}) gallate because of solubility problems of the esters having more than 14 carbon atoms in the water-based test media. Among the compounds tested unequivocally, dodecyl gallate was found to be the most potent inhibitor. Therefore, the emphasis of current study was centered on dodecyl gallate.

Hexyl gallate was obtained in 56% yield as a colorless solid: ^{1}H NMR (270 MHz) δ 0.88 (t, J = 6.5 Hz, 3H), 1.22−1.46 (m, 6H), 1.65−1.76 (m, 2H), 4.18−4.28 (br, 2H), 6.4−5.8 (br, 3H, exchangeable with D_2O), 7.2 (s, 2H); IR (KBr) 3389, 2932, 1687, 1614, 1448 cm^{-1}.

Octyl gallate was obtained in 87% yield as a colorless solid: ^{1}H NMR (270 MHz) δ 0.88 (t, J = 6.5 Hz, 3H), 1.20−1.48 (m, 13H), 1.64−1.78 (m, 2H), 4.24 (t, J = 6.5 Hz, 2H), 7.15 (s, 2H); IR (KBr) 3325, 2926, 1686, 1614, 1468 cm^{-1}.

Decyl gallate was obtained in 65% yield as a colorless solid: ^{1}H NMR (270 MHz) δ 0.88 (t, J = 6.5 Hz, 3H), 1.22−1.47 (m, 15H), 1.66−1.78 (m, 2H), 5.24 (t, J = 6.5 Hz, 2H), 7.16 (s, 2H); IR (KBr) 3391, 2924, 1686, 1514, 1448 cm^{-1}.

Tyrosinase Assay. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although mushroom tyrosinase differs somewhat from other sources (van Gelder et al., 1997), this fungal source was used for the entire experiment because it is readily available. It should be noted that the commercial tyrosinase was described to contain numerous proteins besides tyrosinase (Kumar and Flurkey, 1991) but was used without further purification. The preliminarily assay of the samples was tested at 167 μg/mL. The samples were first dissolved in DMSO and used for the experiment at 30-fold dilution. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (usually not exceeding 10 min, unless otherwise specified). Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated solutions.

The tyrosinase assay was performed as previously described (Masamoto et al., 1980) with some modification. First, 1 mL of a 2.5 mM L-DOPA or L-tyrosine solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units, added last) were added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm.

The preincubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the samples solution (equivalent amount of ID_{50}), and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units). The mixture was preincubated at 25 °C for 5 min. Then, 0.4 mL of 6.3 mM L-DOPA solution was added, and the reaction was monitored at 475 nm for 2 min.

For the measurement of the visible (350−550 nm) spectra of the oxidation products produced by mushroom tyrosinase, the mixture consisting of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 1.0 mL of water, or 2.5 mM L-DOPA solution was preincubated at 25 °C for 10 min. Subsequently, 0.1 mL (7.5 mM) of the sample solution and 0.1 mL of the aqueous solution of the enzyme (138 units, added last) were added, and the mixture was incubated at 25 °C for 3min. Then, the spectra were recorded.

Autoxidation Assay. Oxidation of linoleic acid was measured as previously described (Osawa and Namiki, 1981). The test samples dissolved in 120 μL were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 2.28 mL of 2.51% linoleic acid in ethanol and 9 mL of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40 °C. At intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 9.7 mL of 75% ethanol, which was followed by adding 0.1 mM of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured.

Table 1. Tyrosinase Inhibitory Activity of Gallic Acid and Its Esters[a]

compounds tested	ID_{50} (mM)
gallic acid	4.5
C_1	0.35
C_3	0.31
C_6	0.21
C_8	0.33
C_{10}	0.28
C_{12}	0.49

[a] With respect to L-DOPA.

Radical Scavenging Activity on DPPH. The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1 mL of ethanol, and 0.5 mL of ethanolic solution of DPPH. After the mixture was allowed to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution (Blois, 1958). Inhibitory activity was expressed as the mean 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration−inhibition curves.

Superoxide Anion Assay. The xanthine oxidase (XOD, EC 1.2.3.2) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Superoxide anion was generated enzymatically by xanthine oxidase system. The reaction mixture consisted of 40 mM sodium carbonate buffer (pH 10.2) containing 0.1 mM xanthine, 0.1 mM EDTA, 50 μg protein/mL bovine serum albumin, 25 mM nitroblue tetrazolium (NBT), and 3.3×10^{-3} units of xanthine oxidase in a final volume of 3 mL. After incubation at 25 °C for 20 min, the reaction was terminated by the addition of 0.1 mL of 6 mM $CuCl_2$. Since superoxide anion reduces yellow NBT to blue formazan, the generation of superoxide anion can be detected by measuring the absorbance of formazan newly produced at 560 nm (Toda et al., 1991).

RESULTS AND DISCUSSION

Tyrosinase Inhibitory Activity. The inhibitory activity of gallic acid (**1**) and its esters differing in their alkyl chain lengths tested with respect to the oxidation of L-DOPA catalyzed by mushroom tyrosinase is listed in Table 1. As opposed to gallic acid, ID_{50} values of all the esters are almost comparable. The bioassay with gallic acid showed a concentration-dependent inhibitory effect on this oxidation, and the ID_{50} was determined as 767 μg/mL (4.5 mM). This ID_{50} is about 7-fold less potent than that of benzoic acid, a well-documented tyrosinase inhibitor (Pifferi et al., 1974; Wilcox et al., 1985; Conrad et al., 1994). In addition, gallic acid itself served as a substrate and was oxidized by the enzyme. This oxidation was characterized by a new peak with the maximum at 383 nm, presumably corresponding to *o*-quinone absorption (Passi and Nazzaro-Porro, 1981), but at a much slower rate without a cofactor. There was a progressive increase in the observed rate of this slow oxidation as soon as catalytic amounts of L-DOPA became available as a cofactor, and a yellowish color was immediately detected optically. As long as a cofactor was present, gallic acid was oxidized faster than L-

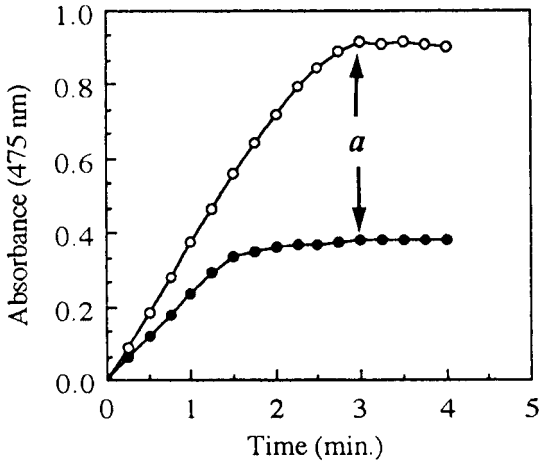

Figure 1. Inhibitory effect on the oxidation of L-DOPA catalyzed by mushroom tyrosinase: (○) without and (●) with gallic acid (4.5 mM).

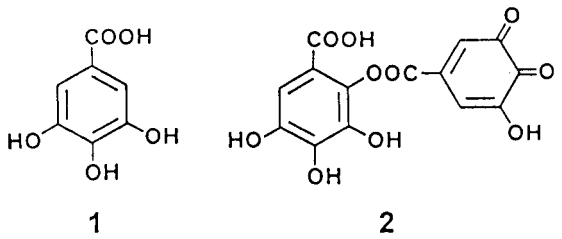

Figure 2. Structure of gallic acid (**1**) and its possible structure of its primary oxidation product (**2**) catalyzed by tyrosinase.

DOPA since the peak at 383 nm reached its plateau faster than that of 475 nm. This may be the reason gallic acid was reported as a tyrosinase inhibitor and also a substrate (Passi and Nazzaro-Porro, 1981; Andrawis and Kahn, 1990; Matsuo et al., 1997). Because of its enzymatically oxidizable nature, the mode of inhibition of gallic acid could not be analyzed. It should be noted that the assay was carried out in air-saturated aqueous solutions. Therefore, after several minutes, dopachrome formation reached the plateau as all the available oxygen in the cuvette was consumed. As shown in Figure 1, the inhibitory curve lowered compared to the control curve and the difference of "*a*" indirectly demonstrates the amount of oxygen in the cuvette used for the oxidation of gallic acid. The oxidation products are a complex mixture of polar compounds, and the unstable nature of the intermediates makes their characterization difficult. Despite our efforts, an attempt to characterize them failed. However, the resulting *o*-quinone may condense with one another through a Michael-type addition, yielding a quinol−quinone product (**2**) (Sayre and Nadkarni, 1994) (Figure 2), though the possibility that the *o*-quinone may form adducts with different nucleophilic groups in the enzyme and inactivates it cannot be entirely ruled out. The former case seems to be more likely since the remaining L-DOPA in the cuvette was oxidized when oxygen was supplied by mixing as illustrated in Figure 3. This result indicates that the enzyme was not inactivated by a K_{cat}-type inhibition (inactivation of the enzyme by products of the reaction), as long as the current experiment is concerned. It appears that gallic acid was isolated as a tyrosinase inhibitor by bioassay-guided fractionation using mushroom tyrosinase similar to the previous report (Matsuo et al., 1997), but this phenolic acid can

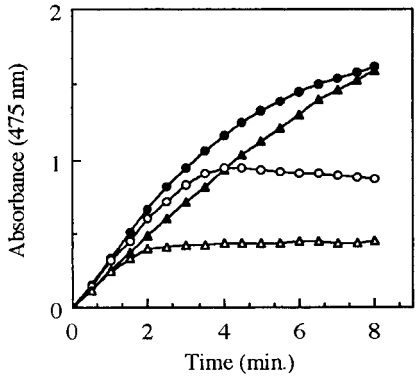

Figure 3. Mixing effect of gallic acid (4.5 mM): (○) without gallic acid; (●) without gallic acid and with mixing; (△) with gallic acid; (▲) with gallic acid and with mixing.

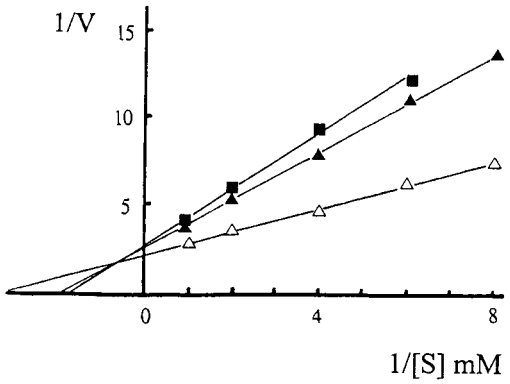

Figure 4. Lineweaver−Burk plots of mushroom tyrosinase and L-DOPA without (△) and with dodecyl gallate [(▲) 0.18 mM and (■) 0.3 mM]. 1/*V*: 1/Δ475 nm/min.

also be termed as an alternate substrate if enough oxygen is supplied.

Similarly, methyl (C_1) gallate was characterized as an inhibitor but also served as an alternate substrate. That is, this gallate was oxidized by the enzyme at a slow rate and yielded the colored oxidation products. This slow oxidation rate was also significantly increased when catalytic amounts of L-DOPA became available as a cofactor. Similar to those observed with gallic acid, the difference of *a* in the inhibition curve of methyl gallate indirectly demonstrates the amount of oxygen in the cuvette used for the oxidation of methyl gallate. However, in contrast to gallic acid, the oxidation products of methyl gallate did not show noticeable absorption around 380 nm. It is obvious therefore that the oxidation products, a complex mixture of polar compounds, were different from those of gallic acid, presumably the resulting *o*-quinone immediately undergoes a series of nonenzymatic reactions. In addition, similar results were also obtained with up to nonanyl (C_9) gallates, though yields of the yellowish oxidation products were decreased with increasing the carbon number.

Interestingly, dodecyl (C_{12}) gallate inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase, but without yielding the colored products. The ID_{50} was determined as 147 μg/mL (0.49 mM), which is nearly 11-fold more potent compared to that of gallic acid. The inhibition kinetics of dodecyl gallate was analyzed by a Lineweaver−Burk plot as shown in Figure 4. The result indicates that dodecyl gallate exhibited as a mixed-type inhibitor with respect to the oxidation of L-DOPA catalyzed by mushroom tyrosinase. In addition, preincubation of the enzyme in the presence of 0.49 mM of

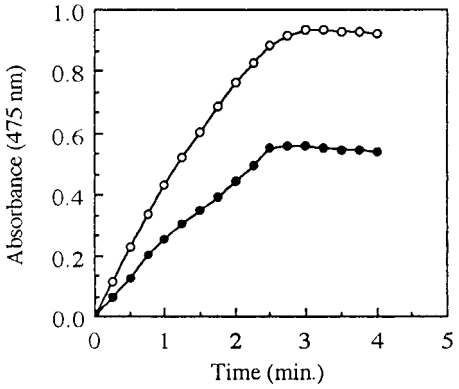

Figure 5. Inhibitory effect on the oxidation of L-DOPA catalyzed by mushroom tyrosinase: (○) without and (●) with dodecyl gallate (0.49 mM).

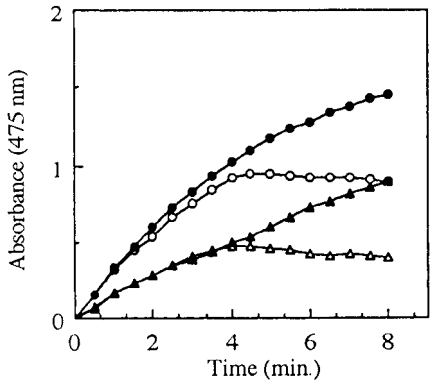

Figure 6. Mixing effect of dodecyl gallate (0.49 mM): (○) without dodecyl gallate; (●) without dodecyl gallate and with mixing; (△) with dodecyl gallate; (▲) with dodecyl gallate and with mixing.

dodecyl gallate and in the absence of L-DOPA did not decrease the enzyme activity significantly. Similar to those observed with the short alkyl chain esters aforementioned, the inhibition curve of dodecyl gallate indicates that the oxygen in the cuvette was used in part for the oxidation of dodecyl gallate as illustrated in Figure 5. This observation may indicate that dodecyl gallate was oxidized to the corresponding *o*-dihydroxy derivative by the enzyme without further oxidation to the yellowish *o*-quinone. The resulting *o*-quinone usually undergoes secondary reactions, either between quinones or with other substances in the immediate vicinity, and a variety of colored compounds are formed consequently (Janovitz-Klapp et al., 1990). In the current experiment, the pigmented products did not appear up to 2 h, indicating dodecyl gallate was not oxidized to the corresponding *o*-quinone. The alternative possibility that dodecyl gallate is oxidized to the *o*-quinone, which oxidizes other products with lower redox potentials, and is immediately reduced back to the colorless *o*-dihydroxy derivative cannot be totally ruled out because tyrosinase usually oxidizes *o*-diphenols to *o*-quinones. However, this possibility is unlikely. Or, the resulting *o*-quinone inactivates the enzyme without producing the pigmented products, but this possibility is even more unlikely. Most probably, dodecyl gallate is not oxidized under the current experimental conditions and inhibits the oxidation of L-DOPA, presumably by being coordinated to copper as a substrate analogue and positioned over the binuclear site (Winkler et al., 1981; Conrad et al., 1994). This likely suggests that dodecyl gallate is an inhibitor rather than an inactivator of the enzyme (Kahn and Andrawis, 1985). The above assumption can be supported by the observation that the remaining L-DOPA in the cuvette was oxidized when oxygen became available by mixing as shown in Figure 6. The inhibition activity exerted by dodecyl gallate could be, at least in part, on the basis of the assumption that the corresponding oxygen *a* can be held as the peroxide in the *oxy*-form of tyrosinase [$\mathbf{E_{oxy}}$] to which dodecyl gallate binds as an inhibitor [**I**] and the resulting peroxo-inhibitor complex [$\mathbf{E_{oxy}I}$] is inactive. On the basis of the above observation, it may be reasonable to conclude that the gallates with increasing the carbon number of the alkyl group ($>C_{10}$) may become harder to be embraced by the protein pocket (Tanford, 1980; Wilcox et al., 1985) and as a result decrease the rate of oxidation by the enzyme. Hence, the gallates with the longer alkyl group ($>C_{10}$) become inhibitors but not substrates. This indicates that the gallates with the longer alkyl group ($>C_{10}$) can be expected as more appropriate inhibitors. However, tetradecanyl gallate (C_{14}) and hexadecanyl gallate (C_{16}) tested are hardly soluble in the water-based test media. This caused variation with O.D. readings that were essential in determining the ID_{50}. Therefore, their ID_{50} could not be established unequivocally. The precise explanation for the role of the alkyl chain length—which must be related to their tyrosinase inhibitory activity—still remains obscure. However, it is apparent that the oxidation rate of the gallates by the enzyme decreases with increasing the alkyl chain length. In other words, the gallates with increasing hydrophobicity of the molecules become more resistant to being oxidized by the enzyme. On the basis of this observation, it may not be illogical to assume that more hydrophobic interaction with the enzyme increases disruption of the tertiary structure of the enzyme and increases the resistance to being oxidized.

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monophenolase and as an *o*-diphenolase (Lerch, 1987; Sánchez-Ferrer et al., 1995). The discussion so far described is, however, on the basis of the experiment using L-DOPA as a substrate. Therefore, the activity aforementioned is *o*-diphenolase inhibitory activity of mushroom tyrosinase. In the absence of a cofactor, the hydroxylation of monophenols such as L-tyrosine to L-DOPA is characterized by an initial lag period. Some reductants added exogenously can either reduce or abolish the lag period (Prota, 1992). *o*-Dihydroxyphenols have been described as the most efficient cofactors for the hydroxylation of monophenols (Pomeranz and Warner, 1967). Interestingly, the lag period for the hydroxylation of L-tyrosine was eliminated in the presence of pyrogallol, but not gallic acid. This indicates that gallic acid does not serve as a cofactor. It is worthwhile to add that the gallate esters, regardless of their carbon chain length, slightly shorten the lag time, indicating they serve as a weak cofactor.

As far as the ID_{50} values are compared, the alkyl chain length of the gallates seems to be not related to their mushroom tyrosinase inhibitory activity. However, gallic acid and its short alkyl ($<C_{10}$) chain esters were oxidized by tyrosinase as substrates yielding the yellow oxidation products, but the long alkyl ($>C_{10}$) chain esters inhibited the enzyme without producing the pigmented products, indicating that the carbon chain length is related to their tyrosinase inhibitory activity in different ways. Although gallic acid and its esters

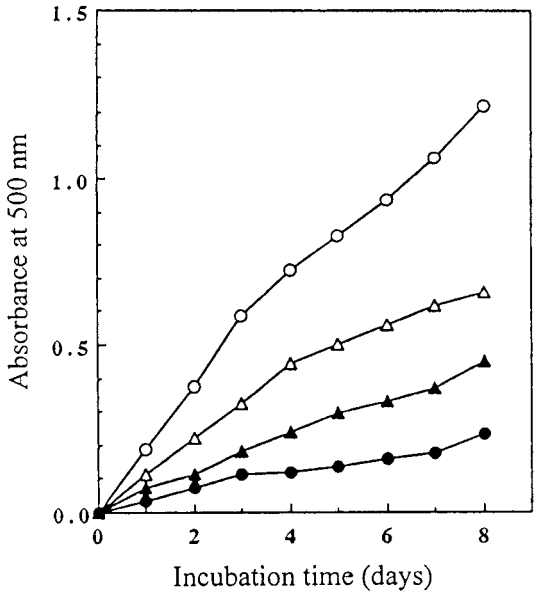

Figure 7. Antioxidative activity of (▲) gallic acid, (●) dodecyl gallate, (△) α-tocopherol, and (○) control. Each compound was added at a final concentration of 30 μg/mL.

were reported as substrates of mushroom tyrosinase (Passi and Nazzaro-Porro, 1981), the long alkyl ($>C_{10}$) chain esters can be used as tyrosinase inhibitors since they do not yield the pigmented products. A similar result was recently reported that the long alkyl ($>C_{12}$) chain gallates prevent cell damage induced by hydroxyl radicals or hydrogen peroxides (Masaki et al., 1997). A more hydrophobic alkyl ($>C_{12}$) chain gallates may provide the unique ability to be positioned in the cell membrane, similar to the phytyl chain in tocopherols and tocotrienols (Papas, 1999). It appears that the current study using mushroom tyrosinase is a simple but effective strategy for the initial screening to search for antibrowning agents. Needless to add, other experimental factors such as the reaction time and amount of oxygen are needed to be considered from a practical point of view. In connection with this, the previous report that mushroom tyrosinase differs somewhat from other sources (van Gelder et al., 1997) also needs to be borne in mind.

Antioxidant Activity. Unsaturated fatty acids, especially linoleic acid, are the target of lipid peroxidation. The effect of gallic acid and dodecyl gallate on autoxidation of linoleic acid was measured by the ferric thiocyanate method as previously described (Osawa and Namiki, 1981). The result is shown in Figure 7. In a control reaction, the production of lipid peroxide increased almost linearly during 8 days of incubation. α-Tocopherol, also known as vitamin E, at 30 μg/mL inhibited the linoleic acid peroxidation almost 50%. Both gallic acid and dodecyl (C_{12}) gallate were found to be more effective in preventing this oxidation than α-tocopherol. About 50% inhibition was observed at 10 μM with dodecyl gallate. Dodecyl gallate was found to show more potent activity than gallic acid, indicating the dodecyl group is not essential but related to the activity.

All the esters tested, regardless of their carbon chain length, exhibited antioxidant activity as expected. Membrane lipids are abundant in unsaturated fatty acids. These unsaturated molecules are most susceptible to oxidative processes, particularly linoleic acid. Lipid peroxidation is a typical free-radical oxidation and proceeds via a cyclic chain reaction (Witting, 1980). The

Table 2. Antioxidative Activity of Gallic Acid and Its Esters

	IC_{50} (μM)	
gallates tested	DPPH radical	$O_2^{\bullet -}$ generation
gallic acid	2.86	5.94
C_1	3.73	9.51
C_2	4.41	10.30
C_3	4.23	11.49
C_6	4.82	25.15
C_8	6.39	>100
C_{10}	4.82	>100
C_{12}	6.39	>100

radical scavenging activity, which can be measured as the decolorizing activity following the trapping of the unpaired electron of DPPH, was examined. Gallic acid and its esters assayed showed almost equally potent radical scavenging activity. The complete scavenging activity was obtained in the range of 10−30 μM, and their 50% scavenging concentrations are listed in Table 2.

It is well-established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. The one-electron reduction products of O_2, superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxy radical ($HO^{\bullet}$) actively participate in the initiation of lipid peroxidation. Several oxidative enzymes, such as xanthine oxidase, produce the $O_2^{\bullet -}$ radical as a normal product of the one-electron reduction of oxygen, resulting in tissue injury (Mayumi et al., 1993). The scavenging activity of gallic acid and its esters against enzymic-generated superoxide anion by xanthine oxidase system is shown in Table 2. Among them, gallic acid exhibited the most potent scavenging activity with IC_{50} of 5.9 μM, and the long alkyl ($>C_{10}$) chain gallates did not show any activity up to 100 μM, indicating that the alkyl chain length is not associated with this activity.

SUMMARY

In addition to their autoxidation inhibitory activity, all the gallate esters tested, regardless of their carbon chain length, showed potent scavenging activity on the DPPH radical, indicating that the alkyl chain length is not related to this activity. Therefore, all these esters can be considered as antioxidants. The result is in agreement with the current use of gallates—propyl, octyl, and dodecyl—as antioxidant additives in foods and previous report (Gunckel et al., 1998). If the gallates need to be used only as antioxidants, the selection depends on their physical properties, such as boiling point and solubility. It should be kept in mind, however, that methyl and propyl gallates were reported to exert prooxidant effects toward DNA and carbohydrates by interacting with iron (Aruoma et al., 1993). In addition, the short alkyl ($<C_{10}$) chain esters were oxidized themselves by tyrosinase and produced the pigmented oxidation products, so it appears that the long alkyl ($>C_{10}$) chain gallates can be considered as more applicable tyrosinase inhibitors with antioxidant activity. The complete scavenging activity of gallic acid was observed in the range of 10−30 μM that is much less than the amounts needed to use as a tyrosinase inhibitor. This potent scavenging activity should be of considerable advantage to control enzymatic pigmentation, since radicals are involved in melanin synthesis. For example, superoxide anion was reported to enhance the oxidation

rate of L-tyrosine to dopachrome by tyrosinase (Wood and Schallreuter, 1991).

As a food additive, the long alkyl ($>C_{10}$) chain gallates act as an antioxidant and tyrosinase inhibitory agent in food and prevent the browning process caused enzymatically and nonenzymatically. After the gallates are consumed together with the food to which they are added as additives, the esters are hydrolyzed, at least in part, to the original gallic acid and the corresponding alcohols that are common plant components. It is worthwhile to note that the liberated gallic acid still acts as a potent antioxidant; for example, it scavenges superoxide anion generated in living systems. Gallic acid showed the most potent scavenging activity against enzymic-generated superoxide anion by the xanthine oxidase system, as well as the DPPH radical. It may be useful for protecting oxidative damages in living systems. There is evidence that antioxidants are significantly associated with reduced cancer risks (Yagi, 1987) and other diseases (Garewal, 1997). The primary biological role of antioxidants is in preventing the damage that reactive free radicals can cause to cells and cellular components. Therefore, the antibrowning gallate esters may be considered even as a cancer fighting diet supplement or chemopreventive agent. This may be supported by the recent report that gallic acid induced apoptotic cell death in human promyelocytic leukemia HL-60 cells (Sakagami et al., 1997). However, the relevance of the results of in vitro experiments in simplified systems to the in vivo situation should be carefully considered when designing antioxidants for addition to foods.

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