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Effect of Polyphenols on Oxymyoglobin Oxidation: Prooxidant Activity of Polyphenols in Vitro and Inhibition by Amino Acids

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ABSTRACT: Effects of various plant phenolics, including polyphenols, on the oxidation of oxymyoglobin were investigated. Most phenolics promoted the oxidation of oxymyoglobin at both pH 5.4 and 7.4. Potent oxidation-promoting activity was observed by several efficient antioxidant polyphenols with a catechol moiety. Therefore, effects of the catechol structure were investigated using dihydrocaffeic acid analogues. The results clarified that ortho- or para-substituted diphenol structures were important for promoting the oxidation of oxymyoglobin. Inhibition of such prooxidant activity for oxymyoglobin by dihydrocaffeic acid was also investigated. Although the required concentration was relatively higher than that of dihydrocaffeic acid, several amino acids inhibited the oxidation. Among these, cysteine was the most potent. Although cysteine alone completely inhibited oxidation at a concentration above 1 mmol/L, 0.1 mmol/L cysteine showed oxidation-promoting activity. In the presence of 0.1 mmol/L dihydrocaffeic acid, in the range of 0.01 mmol/L to 1 mmol/L cysteine, 0.1 mmol/L cysteine showed the most efficient inhibition. These results suggest the possibility of the formation of some equimolar complexes of dihydrocaffeic acid and cysteine such as 5′-cysteinyl dihydrocaffeic acid, which may be produced during the prooxidation of dihydrocaffeic acid, contributing to the inhibition of the oxidation of oxymyoglobin.

KEYWORDS: *oxymyoglobin oxidation, polyphenol, prooxidant activity, cysteine*

INTRODUCTION

Food colors are important stimulants of human appetite, and various food colors have been developed from natural and industrial sources. The red color of fresh meat originates from the muscle heme protein myoglobin. It is well-known that the color of myoglobin varies with the status of heme. The bright red color of meat is produced by the presence of oxymyoglobin (MbO_2). However, MbO_2 is not very stable and is readily oxidized to metmyoglobin (MetMb). MetMb has a brownish color that reduces the market value of fresh meat. In addition to the color change, the oxidation of MbO_2 produces a superoxide radical from coordinated oxygen, thereby promoting the oxidative deterioration of meat components. Therefore, controlling the oxidation of MbO_2 is important for maintaining meat quality. Until now, various techniques involving the substitution of coordinated oxygen by nitrogen oxide or carbon monoxide have been developed to protect red meat color.[1−3] However, some consumers are afraid of the application of these techniques to fresh meat from the viewpoint of food safety. Although various conditions have been investigated to protect the color change of fresh meat due to the oxidation of MbO_2, storage at a very low temperature remains the only effective method at present. Hence, extensive investigations of the effects of various food components on the oxidation of MbO_2 are required to develop new color preservation technologies for fresh meat.

It is well-known that polyphenols and related phenolics are very potent antioxidant constituents of plant-derived foods. They have various mechanisms for protecting food substances from oxidative deterioration, including radical trapping, removal of transition metals, and reducing ability. Ashida and co-workers[4] have reported the inhibition of MbO_2 oxidation with relatively low concentrations of gallic acid similar to that seen with vitamin C. They also investigated the effects of extracts from various edible plants on the oxidation of MbO_2 and reported that some plant extracts inhibited this reaction.[5] However, they have also stated that extracts from some Compositae and Labiatae plants, which they recognized as antioxidative herbs, promote the oxidation of MbO_2. Hayes et al.[6] investigated the effects of some antioxidant phenolics on the oxidation of MbO_2 in a muscle model system, showing that some phenolics dose-dependently inhibited oxidation, whereas others promoted oxidation. Kroll and Rawel[7] also suggested that polyphenols oxidatively reacted with myoglobin.[4] They investigated the modification of myoblobin by electrophoresis, MALDI-TOFMS, and enzymatic hydrolysis techniques. It is well-known that that electrophilic addition of some electrophiles derived from oxidized lipid to MbO_2 initiates the oxidation of MbO_2.[8−11] Therefore, polyphenols or their derivatives may react with MbO_2 and lead to its oxidation. Faustman et al.[3] summarized the inhibitory effects of edible plant extracts on the oxidation of MbO_2 in meat and showed that the inhibitory activity was affected by experimental conditions. Although numerous studies have assessed the effects of food constituents on the oxidation stages of myoglobin,[12−16] the effects of polyphenols remain unclear. In this investigation, we studied the effect of potent antioxidant

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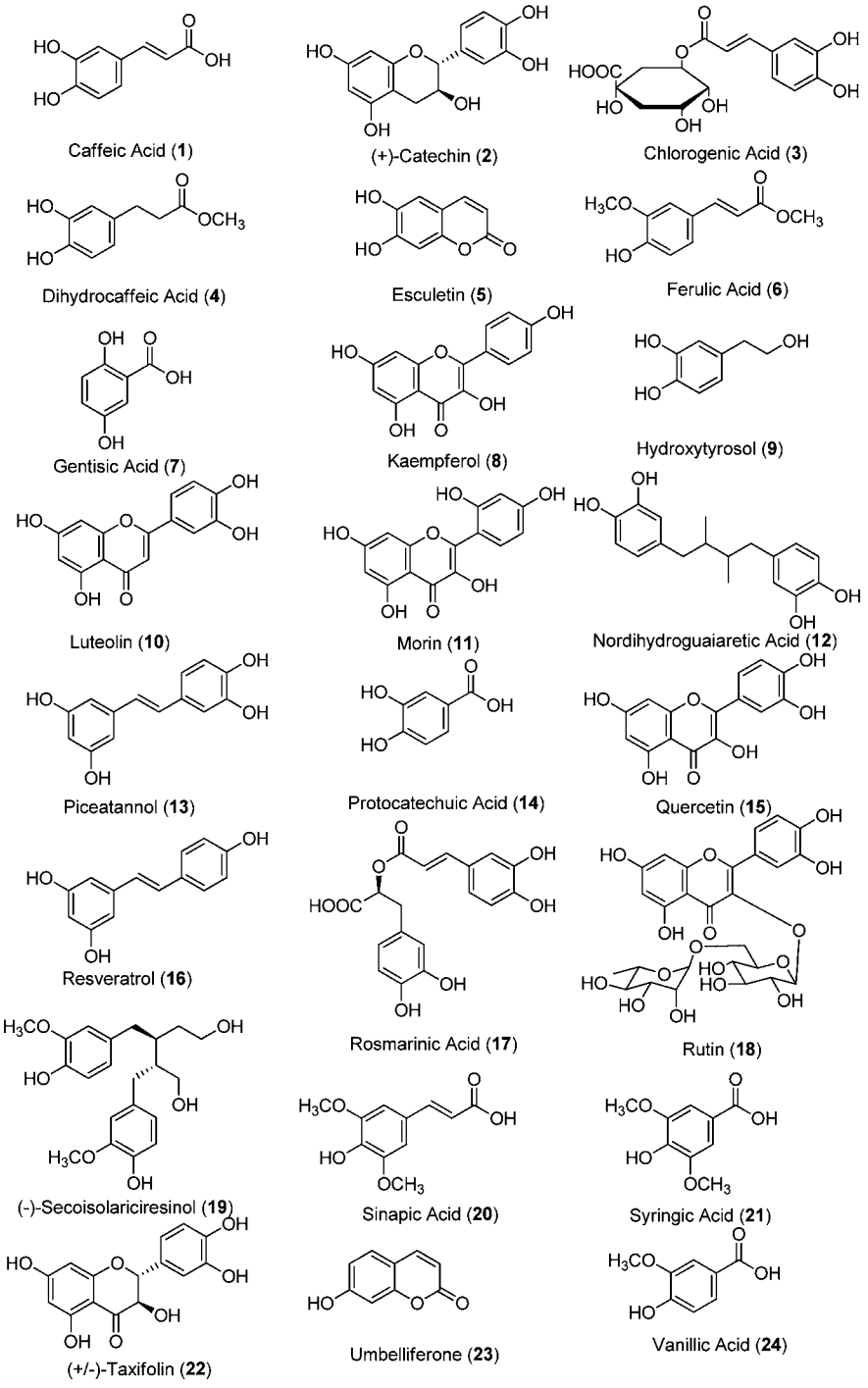

Figure 1. Chemical structures of the investigated plant phenolics that oxidize oxymyoglobin.

polyphenols on the oxidation of MbO_2. Inhibition of the prooxidant activity of the polyphenols by cysteine and other amino acids was also studied.

MATERIALS AND METHODS

Chemicals and Instruments. Myoglobin (from horse heart), quercetin (purity >95% as dihydrate), rutin (purity >95%), gentisic acid (purity >98%), protocatechuic acid (purity >97%), and sodium hydrosulfite were obtained from Nacalai Tesque (Kyoto, Japan). Umbelliferone (purity >98%), caffeic acid (purity >98%), ferulic acid (purity >98%), morin (purity >98% as hydrate), esculetin (purity >98%), sinapic acid (purity >99%), luteolin (purity >98%), hydroxytyrosol (purity >98%), resveratrol (purity >98%), piceatannol (purity >98%), dihydroferulic acid (purity >96%), and homogentisic acid (purity >97%) were purchased from Tokyo Kasei (Tokyo, Japan). (+)-Catechin (purity >98% as hydrate), rosmarinic acid (purity >97%), chlorogenic acid (purity >95%), nordihydroguaiaretic acid (purity >97%), syringic acid (purity >95%), dihydrocaffeic acid (purity >98%), dihydrocoumaric acid (purity >98%), and tyrosinase (from mushroom, 3900 units/mg) were obtained from Sigma-Aldrich (St. Louis, MO). Vanillic acid (purity >96%) was purchased from Wako Pure Chemicals (Osaka, Japan). Kaempferol (purity >97%) and (±)-taxifolin (purity >96%) were obtained from Funakoshi (Tokyo, Japan). (−)-Secoisolariciresinol (purity >95%) was obtained by synthesis.[17] All solvents (extra pure grade or HPLC grade) and amino acids were obtained from Nacalai Tesque. NMR spectra were measured using an ECX-400 spectrometer (JEOL, Tokyo, Japan) with

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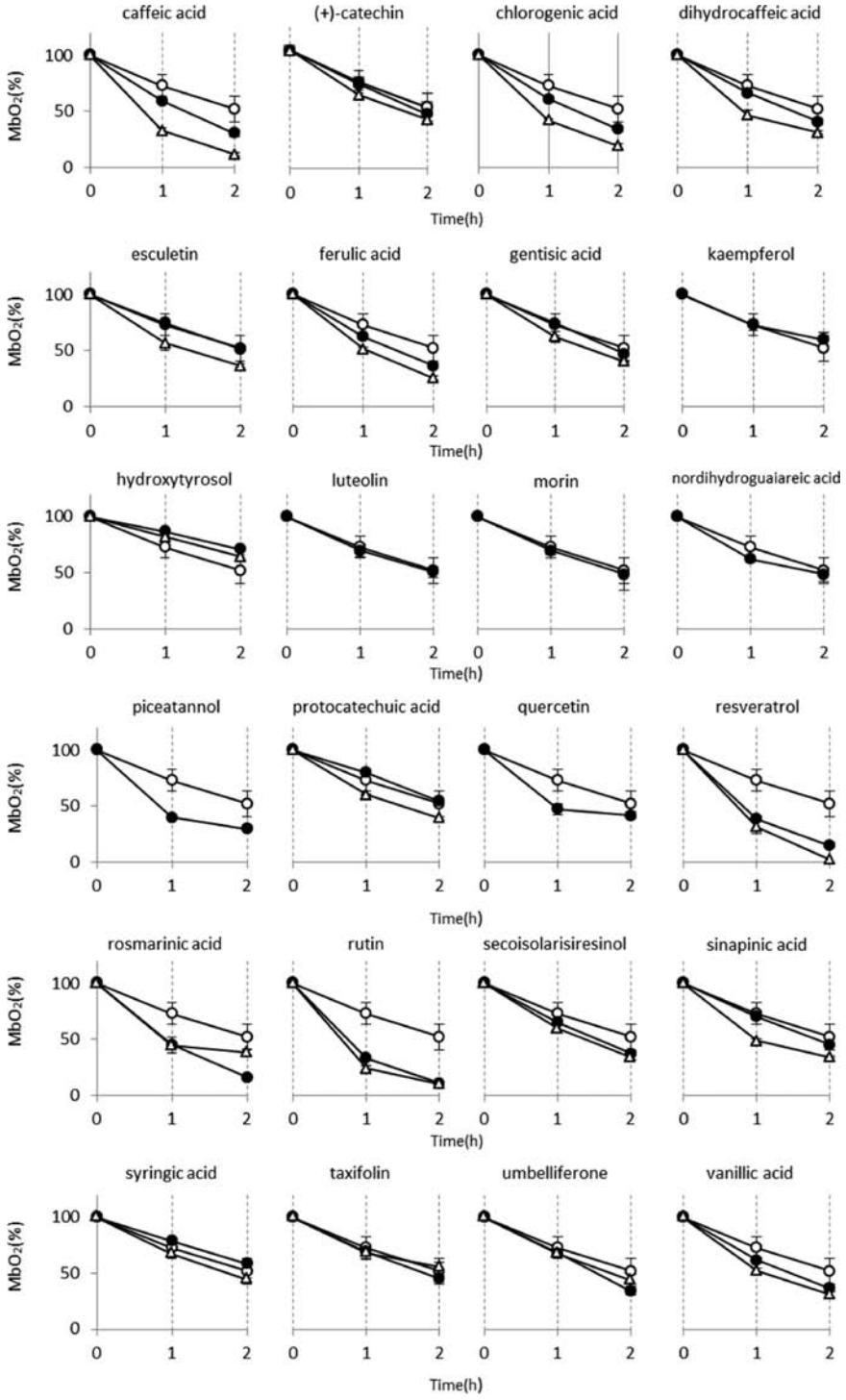

Figure 2. Effects of phenolics on the oxidation of oxymyoglobin under acidic conditions (pH 5.7): ○, without phenolic; ●, with 0.1 mmol/L concentration of each phenolic; △, with 1.0 mmol/L concentration of each phenolic. The initial concentration of oxymyoglobin was 60 μmol/L for all experiments. Data are expressed as mean ± standard deviation (SD) (n = 3). Some phenolics lack 1 mmol/L data because of the poor solubility of these phenolics.

the manufacturer-supplied pulse sequences. High-resolution mass data were obtained using a XEVO QtofMS spectrometer (Waters Japan, Tokyo, Japan) in ESI mode. A PU-2089 low-pressure gradient system (JASCO, Tokyo, Japan) equipped with an MD-2018 photodiode array detector was employed for analytical HPLC. PDA data were analyzed using ChromNAV ver. 1.8 (JASCO). The LC-6AD system (Shimadzu) equipped with a UV-970 detector (JASCO) was used for preparative HPLC.

Preparation of MbO_2. MbO_2 was prepared according to the method reported by Allen and Cornforth[18] with a slight modification. To reduce myoglobin, a myoglobin solution (20 mg in 2 mL) in 50 mmol/L phosphate buffer (pH 7.4) was added to 50 μL of $Na_2S_2O_4$ (0.23 mol/L) in the same buffer at 25 °C. The solution was separated by 10 cm × 1.6 cm i.d. Sephadex G-25 column chromatography (GE healthcare Japan, Hino, Japan). A fine red MbO_2 fraction was collected, and the concentration of MbO_2 was calculated using a spectrophotometer at ε_{582nm} = 15100.[19] The MbO_2 solution

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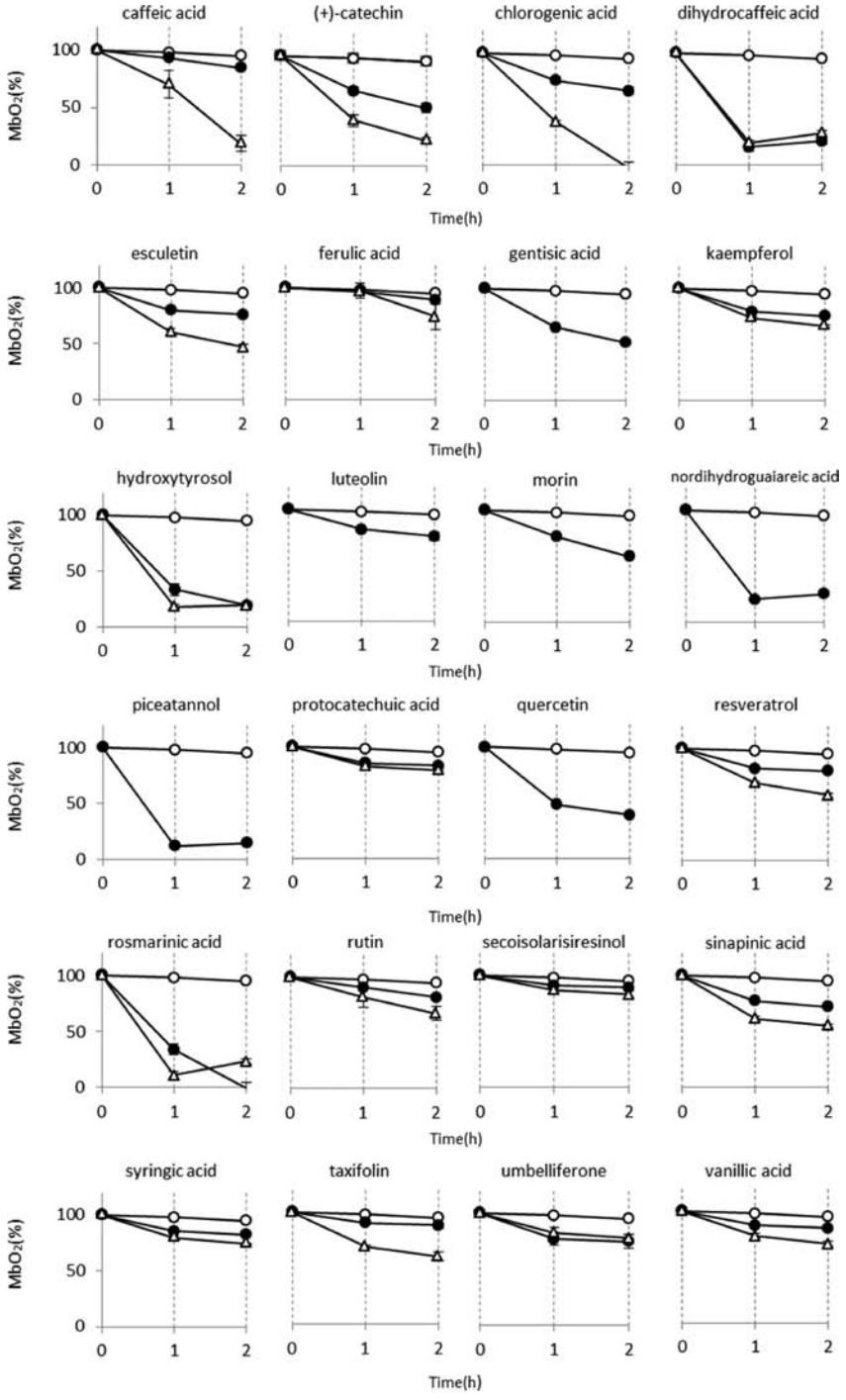

Figure 3. Effects of phenolics on the oxidation of oxymyoglobin under weakly basic conditions (pH 7.4): ○, without phenolic; ●, with 0.1 mmol/L concentration of each phenolic; △, with 1.0 mmol/L of each phenolic. The initial concentration of oxymyoglobin was 60 μmol/L. Some phenolics lack 1 mmol/L data because of the poor solubility of these phenolics.

(approximately 150 μmol/L) was stored at −30 °C after the addition of glycerol (5%) until use.

Oxidation of MbO_2 in the Presence of Phenolics. Stored MbO_2 was washed three times with phosphate buffer (50 mmol/L, pH 7.4) or citrate buffer (50 mmol/L, pH 5.7) using an Amicon Ultra 10K centrifugal ultramembrane filter (Millipore, Billerica, MA) at 14000*g* for 5 min at 4 °C, and the filtered MbO_2 was again dissolved in pH 7.4 or 5.7 buffers to prepare a 60 μmol/L solution. To the MbO_2 solution (300 μL) in a microplate well, 5 μL of the appropriate concentration of test sample in DMSO or the same buffer as the myoglobin solution was added. The oxidation of MbO_2, which is indicated by metomyoglobin formation, was monitored at 570 nm using a model 550 microplate reader (BIO-RAD, Hercules, CA). Oxidation was calculated using the following equation and expressed as residual percent of MbO_2: residual MbO_2 (%) = [initial absorbance of test solution (MbO_2, 60 μmol/L) − measured absorbance of test solution after incubation − absorbance of MetMb (60 μmol/L)] × 100/[initial absorbance of MbO_2 (60 μmol/L) − absorbance of MetMb (60 μmol/L)].

Acetylation of Dihydrocaffeic Acid. Dihydrocaffeic acid (50 mg) was added to acetic anhydride (1 mL) and pyridine (1 mL), and the mixture was kept at 23 °C for 1 day. After acetic anhydride and pyridine were removed in vacuo, the residue was purified by silica gel column chromatography (EtOAc:hexane = 2:3, v/v) containing 1%

acetic acid to give a diacetate of dihydrocaffeic acid, **25** (74 mg): ^{1}H NMR ($CDCl_3$) δ 7.13–7.08 (2H, m, H5′ and 6′), 7.04 (1H, brs, H2′), 2.96 (2H, t, J = 7.7 Hz, H2 or 3), 2.69 (2H, t, J = 7.7 Hz, H2 or 3), 2.28 (6H, s, CH_3); HR-MS (ESI negative) calculated for $C_{26}H_{26}O_{12}Na$ 553.1322, found m/z 553.1274 $[2(M - H) + Na]^-$.

Preparation of a Cysteinyl Dihydrocaffeic Acid. To a mixture of dihydrocaffeic acid (50 mg) and L-cysteine (66 mg) in 50 mmol/L phosphate buffer (pH 6.8, 100 mL) was added tyrosinase (2 mg) at 23 °C. The solution was kept for 0.5 h and was then passed through a Sep-Pak C18 cartridge (Waters Japan). The eluate was concentrated using a freeze-dryer to approximately 20 mL. This solution was subjected to preparative HPLC [a 20 mm × 250 mm i.d., 5 μm, Cosmosil 5C18-AR-II column (Nacalai Tesque); solvent, methanol–1% acetic acid in H_2O (1:9,v/v); flow rate, 7.0 mL/min]. The peak eluted at 49.5 min was collected to give 64 mg of 5′-cysteinyl dihydrocaffeic acid, **26**, after evaporation of the solvent. **26**: ^{1}H NMR (D_2O) δ 6.84 (1H, d, J = 2.0 Hz, H6′), 6.71 (1H, d, J = 2.0 Hz, H2′), 3.61 (1H, dd, J = 9.1 and 3.8 Hz, H3″), 3.61 (1H, dd, J = 14.7 and 3.8 Hz, H2″), 3.06 (1H, dd, J = 14.7 and 9.1 Hz, H2″), 2.69 (2H, brt, J = 7.7 Hz, H2 or 3), 2.51 (2H, brt, J = 7.7 Hz, H2 or 3); HR-MS (ESI negative) calculated for $C_{12}H_{14}NO_6S$ 300.0542, found m/z 300.0549 $[M - H]^-$.

RESULTS AND DISCUSSION

Oxidation of MbO_2 in the Presence of Phenolics. The effects of 24 plant phenolics, including various phenolic acids, flavonoids, and other polyphenols (Figure 1), on the color of MbO_2 were investigated. MbO_2 has a strong absorption maximum around 580 nm, and MetMb (oxidized MbO_2) shows an absorption minimum around 570 nm.[19] Therefore, we used an available 570 nm filter to measure the concentration of MbO_2 using a microplate reader. The oxidation of MbO_2 to MetMb was calculated as a percentage of remaining MbO_2 in the test solution. Results of the oxidation of MbO_2 in the presence of 0.1 mmol/L and 1 mmol/L phenolics under weak acidic conditions (pH 5.7) and under neutral conditions (pH 7.4) are summarized in Figures 2 and 3, respectively. Figure 2 indicates that most phenolics, except hydroxytyrosol, exhibited more rapid oxidation of MbO_2 than the control. It is well-known that acidic conditions affect the stability of MbO_2;[20] therefore, the concentration of MbO_2 in the control (no additive) was reduced to approximately 50% with relatively high variation during 2 h at 37 °C. On the other hand, MbO_2 is more stable in neutral and basic solutions.[21] Therefore, we reinvestigated the effects of these phenolics on MbO_2 at pH 7.4 (Figure 3). Although MbO_2 in the absence of phenolics was very stable for 2 h, in the presence of nonenal (1 mmol/L), an oxidation-enhancing compound,[8] a 46% decrease in the concentration of MbO_2 was observed after 5 h. Most phenolics (0.1 and 1 mmol/L) oxidized MbO_2 in 2 h (Figure 3). Potent oxidation was observed with dihydrocaffeic acid, **4**, hydroxytyrosol, **9**, nordihydroguaiaretic acid, **12**, piceatannol, **13**, quercetin, **15**, and rosmarinic acid, **17**. Notably, these phenolics are well recognized as potent antioxidant polyphenols. Very potent antioxidant polyphenols sometimes act as prooxidants because of their strong oxidizable property as a result of their catechol structures that have a strong reducing potential.[22] In the present study, this property may have led to the rapid oxidation of MbO_2.

Effect of Phenolic Hydroxyl Group on Oxidation of MbO_2. To further investigate structural effects of prooxidant polyphenols, dihydrocaffeic acid, **4**, was selected because it had the simplest structure. To examine the effects of the phenol groups of dihydrocaffeic acid, which may contribute both antioxidant and prooxidant activities, the two phenol groups of dihydrocaffeic acid were acetylated to give a diacetate, **25**. The oxidation of MbO_2 in the presence of the diacetate was measured at pH 7.4; however, diacetate did not show any oxidation effect on MbO_2 during the course of the study (Figure 4). The effect of three analogues of dihydrocaffeic acid

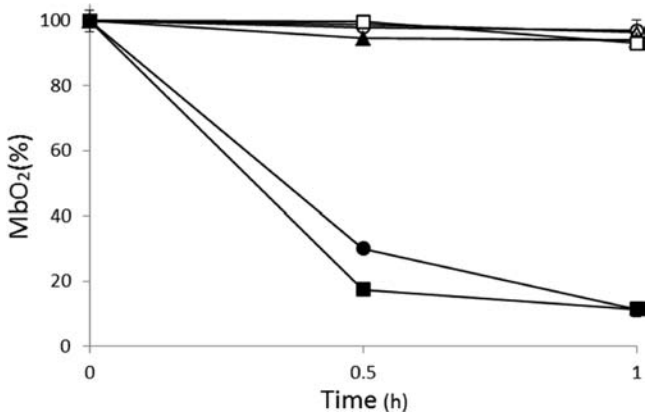

Figure 4. Effects of the phenol group of dihydrocaffeic acid analogues (0.1 mmol/L) on oxidation of oxymyoglobin: ○, without phenolic; ●, dihydrocaffeic acid; △, diacetate of dihydrocaffeic acid (**25**); ▲, dihydroferulic acid; □, dihydrocoumaric acid; ■, homogentistic acid. The initial concentration of MbO_2 was 60 μmol/L for all experiments. Data are expressed as mean ± standard deviation (SD) (n = 3).

on the oxidation of MbO_2 was also examined (Figure 4). Notably, two monophenolic analogues, dihydrocoumaric acid and dihydroferulic acid, did not accelerate the oxidation of MbO_2. However, homogentisic acid, which was employed as a *p*-diphenol analogue of dihydrocaffeic acid, showed very rapid oxidation activity for MbO_2 similar to that of dihydrocaffeic acid. It is well-known that the phenolic hydroxyl group is essential for radical trapping from oxidative intermediates of oxidizing food compounds. The antioxidant activity of monophenol compounds is not very strong, whereas that of *o*- and *p*-diphenol compounds is always strong in vitro because of the easy formation of stable quinones or semiquinone radicals from these compounds. The quinones and semiquinone radicals of these *o*- and *p*-diphenols can be produced under air oxidation conditions, and the oxidation of food compounds by these oxidized phenolic derivatives is recognized as prooxidant activity. The oxidation of catechol polyphenols produces *o*-quinone, and the quinone has electrophilic activity. Notably, 4-hydroxynonenal, a typical nonquinone electrophilic compound derived from the oxidative decomposition of lipids, reacted with a histidine residue (imidazole ring) of myoglobin, resulting in the instability of MbO_2.[9,11,21] Polyphenol quinones have similar electrophilic properties because of their α,β-enone structures.[23] When polyphenols act as prooxidants by a mechanism similar to that of aldehydes, MbO_2 can be protected from oxidation by external histidine or related nucleophilic amino acids.

Effect of Amino Acids on the Oxidation of MbO_2 Caused by Dihydrocaffeic Acid. The effect of coexisting histidine on dihydrocaffeic acid (0.1 mmol/L)-mediated oxidation of MbO_2 was examined. Although one equivalent concentration of histidine did not inhibit oxidation, higher concentrations (>1 mmol/L) significantly inhibited oxidation (40% inhibition with 2 mmol/L histidine). The effects of other amino acids were also investigated at the same concentration of

the histidine (Figure 5). Although most amino acids did not inhibit oxidation very significantly, cysteine showed much

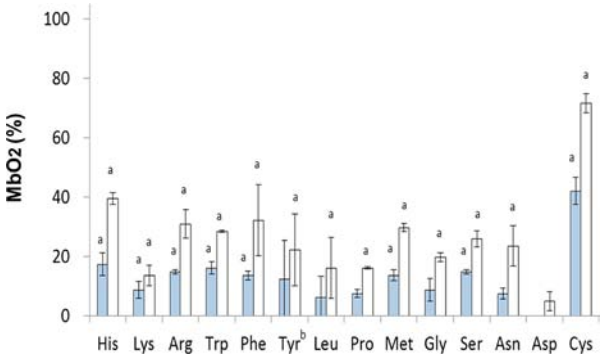

Figure 5. Effect of amino acids on dihydrocaffeic acid (0.1 mmol/L)-induced oxidation of oxymyoglobin. Concentration of amino acids: shaded bars, 1 mmol/L; open bars, 2 mmol/L. The initial concentration of oxymyoglobin was 60 μmol/L for all experiments. Data were expressed as mean ± standard deviation (SD) (n = 3). a: Statistical significance (P < 0.05) is observed from the data of control experiments (with only dihydrocaffeic acid) by Student's t-test. b: The solution was slightly turbid.

higher inhibition. Cysteine has a thiol group, and thiols possess both potent nucleophilicity and radical reactivity,[22,24,25] which can interact with quinone and semiquinone radicals of dihydrocaffeic acid, respectively. Tang and co-workers[26] have investigated the effect of glutathione, a thiol-bearing peptide, on the oxidation of MbO_2 and showed its prooxidant activity toward equine MbO_2. However, whether cysteine promotes or inhibits the oxidation of MbO_2 remains unclear. Figure 6 shows concentration effects of dihydrocaffeic acid, cysteine, and cysteine with 0.1 mmol/L dihydrocaffeic acid, on the oxidation of MbO_2. Strong prooxidant activity of dihydrocaffeic acid was observed at concentrations ≥0.01 mmol/L (Figure 6A). In contrast, the most potent prooxidation activity of cysteine was observed at the concentration of 0.1 mmol/L. Interestingly, concentration-dependent prooxidant activity of cysteine was observed below 0.1 mmol/L; however, higher concentrations of cysteine (>1 mmol/L) had no effect on the oxidation of MbO_2 (Figure 6B). Figure 6C shows the prooxidant activity of dihydrocaffeic acid (0.1 mmol/L) in the presence of various concentrations of cysteine. A bell-shaped response curve was observed around 0.1 mmol/L cysteine, and concentration-dependent inhibition was also observed above 1 mmol/L. These results revealed that cysteine inhibits the oxidation of MbO_2 at higher concentrations and promotes oxidation at lower concentrations. These data also indicate that an equimolar mixture of dihydrocaffeic and cysteine may form a complex that inhibits the oxidation of MbO_2. Romero and co-workers[27] investigated the effects of several biological thiols on different redox stages of myoglobin; however, they observed that the thiols are not involved in the redox reaction between MetMb and MbO_2, except for lipoic acid. Their results indicated that the observed inhibition may be the effect of a cysteine−dihydrocaffeic acid complex without myoglobin. Unfortunately, we did not find this complex in our reaction solution. When the oxidation of MbO_2 is initiated, oxidation in the system is too rapid to detect any compounds using HPLC because the oxidation of MbO_2 continuously produces superoxide radicals, which in turn might decompose any phenolic compounds.[28]

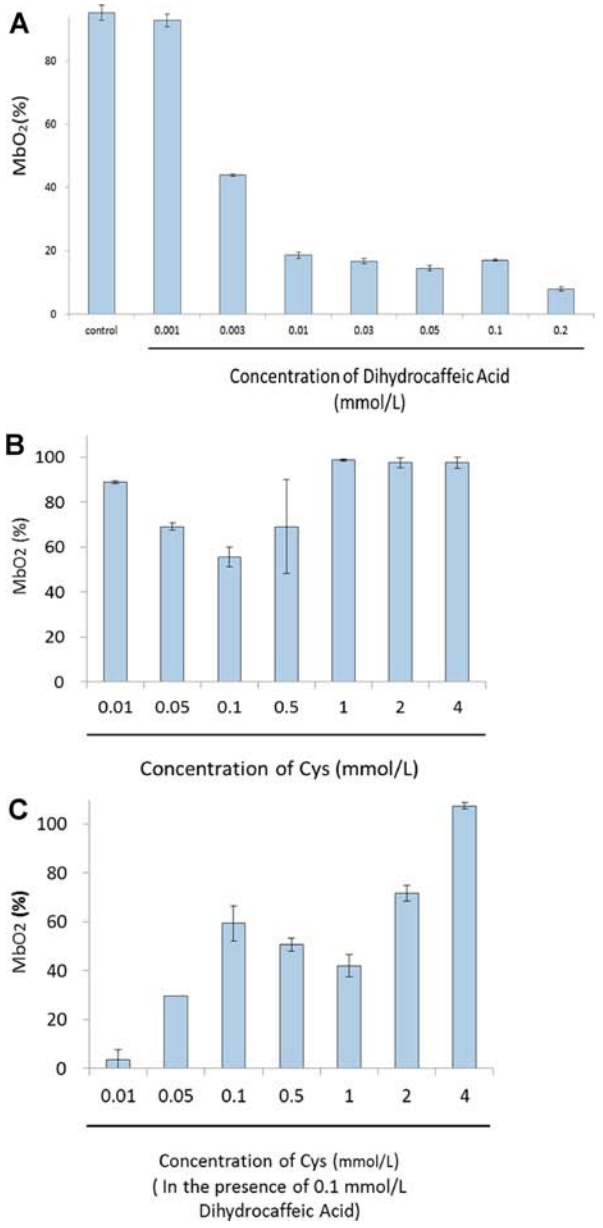

Figure 6. (A) Concentration effects of dihydrocaffeic acid on the oxidation of oxymyoglobin. (B) Concentration effects of cysteine on the oxidation of oxyglobin. (C) Concentration effects of cysteine in the presence of dihydrocaffeic acid (0.1 mmol/L). The initial concentration of oxymyoglobin was 60 μmol/L for all experiments. Data are expressed as mean ± standard deviation (SD) (n = 3).

Effects of a Cysteinyl Dihydrocaffeic Acid on the Oxidation of MbO_2. Catechol polyphenols are powerful antioxidants and are easily oxidized in air. Protein thiol groups such as glutathione and cysteine can react with polyphenols under oxidizing conditions.[29−34] Subsequent formation of cysteine−dihydrocaffeic acid complexes may reduce the prooxidant activity of dihydrocaffeic acid. Moridani et al.[35] have reported that dihydrocaffeic acid formed a 5′-*S*-glutathione-substituted derivative of dihydrocaffeic acid after tyrosinase oxidation. Based on their method, we synthesized 5′-*S*-cysteinyl dihydrocaffeic acid, **26** (Figure 7), and investigated its prooxidant activity toward MbO_2 (Figure 8). As shown in Figure 8, cysteinyl dihydrocaffeic acid does not have any prooxidant activity at concentrations between 0.01 mmol/L

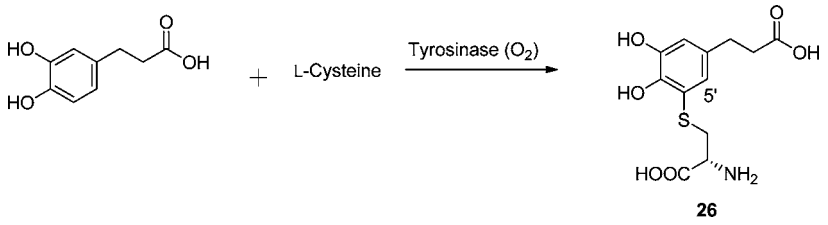

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Figure 7. Synthetic route of cysteinyl dihydrocaffeic acid (**26**).

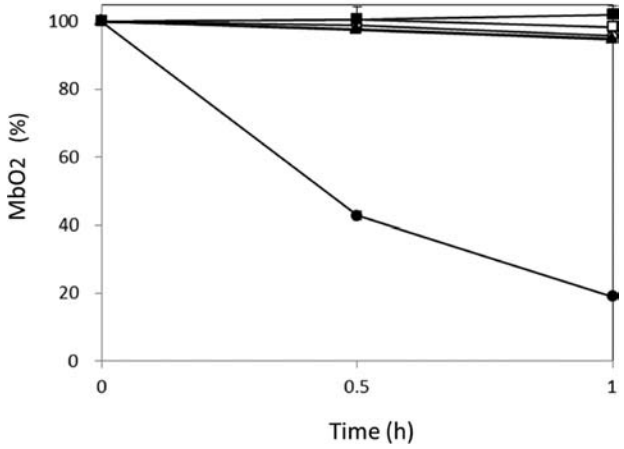

Figure 8. Effect of cysteinyl dihydrocaffeic acid (**26**) on the oxidation of oxymyoglobin: ○, without phenolic; ●, dihydrocaffeic acid (0.1 mmol/L); △, cysteinyl dihydrocaffeic acid (0.01 mmol/L); ▲, cysteinyl dihydrocaffeic acid (0.05 mmol/L); □, cysteinyl dihydrocaffeic acid (0.1 mmol/L); ■, cysteinyl dihydrocaffeic acid (0.5 mmol/L). The initial concentration of MbO_2 was 60 μmol/L for all experiments. Data are expressed as mean ± standard deviation (SD) (n = 3).

and 0.5 mmol/L, whereas 0.1 mmol/L dihydrocaffeic acid has strong prooxidant activity toward MbO_2. Although the contribution of thiol substituents to the prooxidant activity of antioxidant polyphenols remains unclear from the present results, thiol substitution to dihydrocaffeic acid may play important roles in protecting MbO_2 from prooxidation by dihydrocaffeic acid.

We found in this investigation that potent antioxidant polyphenols showed remarkable prooxidant activity for the oxidation of MbO_2, whereas weaker antioxidant polyphenols did not show any prooxidant activity. We also found that such prooxidant activity of antioxidant polyphenols was inhibited by cysteine and other amino acids. Although this investigation was carried out in an in vitro model system and real meat contained various other constituents, which may have an effect on the oxidation of MbO_2 by polyphenols, our findings should provide valuable fundamental information for the effect of antioxidative polyphenols on the oxidative color change of meat products.

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Notes

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