Food Chemistry 138 (2013) 1483–1492

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical evidence for the synergistic effect of a cysteinyl thiol on the antioxidant activity of caffeic and dihydrocaffeic esters

Aya Fujimoto, Miyuki Inai, Toshiya Masuda *

Graduate School of Integrated Arts and Sciences, University of Tokushima, Tokushima 770-8502, Japan

ARTICLE INFO

Article history:
Received 16 August 2012
Received in revised form 12 November 2012
Accepted 15 November 2012
Available online 24 November 2012

Keywords:
Antioxidant activity
Synergism
Cysteinyl thiol
Caffeic acid
Dihydrocaffeic acid

ABSTRACT

Antioxidant activity of methyl caffeate and methyl dihydrocaffeate in the presence of a cysteinyl thiol was measured in an azo-initiator-induced lipid oxidation system. The coexistence of the thiol was observed to display a synergistic effect on the antioxidant activity of both caffeates. The synergism was observed mainly with respect to the elongation of the induction period, rather than the inhibition rate for lipid oxidation. For methyl caffeate, the maximum elongation of the induction period was observed in the presence of more than two equivalents of the thiol, whereas the maximum effect on the activity of methyl dihydrocaffeate was observed in the presence of more than three equivalents of the thiol. These synergistic effects were analysed by high-performance liquid chromatography and liquid chromatography–mass spectrometry analyses of the intermediates produced during the antioxidation period. The analytical results clarified that the mono-thiol adduct of methyl caffeate and the mono- and di-thiol adducts of methyl dihydrocaffeate contributed to the synergism in the antioxidant activity of both caffeates.

1. Introduction

Polyphenols have various useful functions, with respect to not only foods but also human health (Sies, 2010). With an increased knowledge of the functions of polyphenols, various industries have incorporated these compounds into healthy foods, cosmetics and medicines. Although polyphenols have widespread applications, the most important function of polyphenols is still their potent antioxidant activity. It is well known that most of the antioxidant actions of phenolic compounds are based on radical termination reactions. The antioxidation reaction converts polyphenols to various oxidation products *via* polyphenolic radical species (Frankel, 2005). Hence, the antioxidation process should be chemically influenced by surrounding reactive molecules.

Foods are complex systems involving many types of biomolecules. Some food constituents exhibit potential reactivity towards polyphenol radicals and oxidation products of polyphenols. A number of potent antioxidative polyphenols have a catechol moiety in their structures. During the oxidation process, the catechol is converted to *ortho*-quinone *via* a semiquinone radical. Although the quinone does not exhibit any antioxidant activity, it has potential electrophilic properties and can react with surrounding nucleophilic molecules. When addition of the nucleophilic molecules occurs, the catechol structure is regenerated to exhibit antioxidant activity. The semiquinone radicals of polyphenols are also capable of reacting with other radical species, including hydrogen atoms, to regenerate the catechol structure (Masuda et al., 2008).

Niki and co-workers (1984) reported that vitamin E was regenerated with water-soluble vitamin C. Mukai and co-workers (1992) also studied the kinetics of the regeneration of vitamin E by various biological hydroquinones. This regeneration of antioxidative vitamin E is recognised as one of the antioxidant synergisms (Barclay, Locke, & MacNeil, 1983; Mahoney, 1969). Carnosic acid, a potent antioxidative diterpene widely distributed in Labiatae herbs, afforded a non-active quinone as the antioxidation product; however, it can regenerate a catechol structure by the intramolecular addition of its carboxylic acid to recover potent activity (Masuda et al., 2002). Carnosol quinone, an antioxidation product of carnosol, also regenerated a catechol moiety *via* the addition of water (Masuda, Kirikihira, & Takeda, 2005).

Typical nucleophilic molecules in food constituents are amino- or thiol-bearing compounds, such as amino acids, peptides and proteins. Saito and Kawabata (2004) reported that the radical-scavenging ability of protocatechuic acid increased with the addition of a thiol molecule. Rohn, Rawel, and Kroll (2004) demonstrated the antioxidant activity of a coupling product of quercetin, quinone and the amino groups of proteins. If thiol or amino compounds could regenerate the catechol moiety *in situ* during the oxidation reaction of polyphenols, these nucleophilic molecules should exhibit a potent synergistic effect on the antioxidant activity of polyphenols.

* Corresponding author. Fax: +81 88 656 7244.
E-mail address: masuda.ias@tokushima-u.ac.jp (T. Masuda).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.11.073

Previously, we reported the reaction of a cysteinyl thiol with various polyphenols under 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical oxidation conditions (Fujimoto & Masuda, 2012a). The coupling reactions of polyphenols with the thiol are caused by its high nucleophilicity or radical-forming ability; therefore, some polyphenols give di- and tri-thiol adducts in addition to the initially formed mono-thiol adduct (Awad et al., 2002). These results prompted us to investigate the synergistic effect of the thiol on the antioxidant activity of polyphenols. For this purpose, we have focused on the esters of caffeic acid and dihydrocaffeic acid, because they are potent antioxidative polyphenols and are structurally similar to the B and C rings of a flavonoid.

In our previous investigation (Fujimoto & Masuda, 2012a), both caffeates afforded various thiol adducts with a recovered catechol structure. Bassil, Makris, and Kefalas (2005) reported that a cysteine adduct of caffeic acid possessed a stronger radical-scavenging activity but a weaker ferrous-reducing activity in comparison to caffeic acid. In this paper, we report detailed results for the investigation of the synergistic effect of a cysteinyl thiol on the antioxidant activity of both caffeates in a lipid oxidation system.

2. Materials and methods

2.1. Chemicals and instruments

Methyl caffeate (**1**) and methyl dihydrocaffeate (**2**) (each purity > 95%) were synthesised from the corresponding carboxylic acids with methanol in the presence of a catalytic amount of sulphuric acid. Mono-thiol of methyl caffeate (**6**), mono-, di-, and tri-thiol adducts of methyl dihydrocaffeate (**10**, **12**, **13**, respectively), *N*-benzoylcysteine methyl ester (**3**), and *N*,*N*′-dibenzoylcystine dimethyl ester (**4**) were prepared by a previously reported method (Fujimoto & Masuda, 2012a). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was obtained from Wako Pure Chemicals (Tokyo, Japan). Ethyl linoleate was obtained from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel 60 (Merck, Darmstadt, Germany). All solvents (extra pure grade or HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). An LC-20AD low-pressure gradient system (Shimadzu, Kyoto, Japan) equipped with an SPD-M20A photodiode array detector and a DGU-20A3 degasser was employed for analytical HPLC. HPLC data were analysed using LC solution software (ver. 6.10, Shimadzu). A PU-9800 pump equipped with a UV-975 detector (JASCO, Tokyo, Japan) was used for the quantitative analysis of ethyl linoleate hydroperoxides. A XEVO QTOF-MS (Waters Japan, Tokyo, Japan) was used for LC–MS analysis. The sample was injected into the MS instrument through an Acuity UPLC system (Waters) and mass spectra of the observed peaks were obtained under the following conditions; mode, ESI negative; capillary voltage, 2.4 kV; cone voltage, 40 V; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow rate, 50 L h^{-1}; desolvation gas flow rate, 1000 L h^{-1}, MS^E low collision energy, 6 V; MS^E high collision energy, from 20 to 30 V. The elemental composition of each peak compound was calculated from the high-resolution MS data of the protonated or ion-adducted molecular ion using MassLynx software (V. 4.1, Waters).

2.2. Measurement of the antioxidant activity of methyl caffeate and methyl dihydrocaffeate in the presence of a thiol derivative, *N*-benzoylcysteine methyl ester

To ethyl linoleate (70 μL) in a straight vial (75 mm in height, 40 mm in diameter), 200 μL of methyl caffeate or methyl dihydrocaffeate in CH_3CN (25 mM), appropriate amount of *N*-benzoylcysteine methyl ester in CH_3CN, and 500 μL of AMVN in CH_3CN (0.6 M) were added. Each solution was made up to 4 mL with the addition of CH_3CN and then incubated at 37 °C with shaking (100 times min^{-1}) in a water-bath shaker. The control experiment vial was prepared in a similar manner without the addition of methyl caffeate or *N*-benzoylcysteine methyl ester. A portion (20 μL) of the solution was taken from each vial at 1-h intervals and diluted with CH_3CN (380 μL). The diluted solution (10 μL) was injected into the HPLC to analyse ethyl linoleate hydroperoxides under the following conditions; column, YMC-Pak ODS-A (150 × 4.6 mm i.d., YMC, Tokyo, Japan); solvent, CH_3CN/H_2O = 9:1 (v/v); flow rate, 1.0 mL min^{-1}, detection, 234 nm. The antioxidant activity of appropriate amounts of the *N*-benzoylcysteine methyl ester and the activities of methyl caffeate and methyl dihydrocaffeate were also measured under the same conditions. Lipid oxidation was represented as the concentration of ethyl linoleate hydroperoxides, which was obtained using the following equation of the calibration curve of pure ethyl linoleate hydroperoxides; $y = 436{,}497x + 191$ [*y*, concentration of the hydroperoxides (mM); *x*, peak area of the hydroperoxides at 234 nm]. All data were expressed as the mean values obtained from two independent experiments.

2.3. HPLC analysis of the antioxidation products from methyl caffeate and methyl dihydrocaffeate in the presence of the thiol

An aliquot (5 μL) was taken from each antioxidant reaction vial, prepared in the above experiment, at equal intervals and injected into the HPLC column to analyse the reaction products from methyl caffeate or methyl dihydrocaffeate under the following conditions; column, Cosmosil $5C_{18}$-AR-II (250 × 4.6 mm i.d., Nacalai Tesque); solvents, A = 1% acetic acid in H_2O, B = CH_3CN; gradient conditions, linear gradient from 5% of solvent B to 100% of solvent B for 40 min; flow rate, 1.0 mL min^{-1}; detection, 245 and 280 nm. The concentrations of methyl caffeate, methyl dihydrocaffeate and their thiol adducts, which were obtained using each pure sample, were respectively calculated using the following equations: $y = 459{,}000x$ [*y*, peak area of methyl caffeate at 280 nm; *x*, methyl caffeate concentration (mM)], $y = 526{,}000x - 18{,}000$ [*y*, peak area of the mono-thiol adduct at 245 nm; *x*, mono-thiol adduct concentration (mM)], $y = 154{,}000x$ [*y*, peak area of methyl dihydrocaffeate at 280 nm; *x*, methyl dihydrocaffeate concentration (mM)], $y = 428{,}000x - 8500$ [*y*, peak area of the mono-thiol adduct at 245 nm; *x*, mono-thiol adduct concentration (mM)], $y = 721{,}000x - 45{,}000$ [*y*, peak area of the di-thiol adduct at 245 nm; *x*, di-thiol adduct concentration (mM)], $y = 889{,}000x - 51{,}000$ [*y*, peak area of the tri-thiol adduct at 245 nm; *x*, tri-thiol adduct concentration (mM)], $y = 778{,}000x$ [*y*, peak area of *N*-benzoylcysteine methyl ester at 245 nm; *x*, *N*-benzoylcysteine methyl ester concentration (mM)]. All data were expressed as the mean values obtained from two independent experiments.

2.4. Comparison of the reactivity of caffeates and their thiol adducts toward a peroxyl radical

For the comparison of the reactivity between methyl caffeate and its mono-thiol adduct, a mixture of a methyl caffeate solution (5 mM in CH_3CN, 300 μL) and the mono-thiol adduct solution (5 mM in CH_3CN, 300 μL) was prepared and then an AMVN solution (0.6 M in CH_3CN, 300 μL) was added to the solution. The solution was stirred well and then incubated at 37 °C with shaking (100 times min^{-1}) in a water-bath shaker. An aliquot (5 μL) of the solution was injected into the HPLC column to analyse the decrease in the peak area of methyl caffeate and the mono-thiol adduct at 1-h intervals under the following conditions: column, Cosmosil $5C_{18}$-AR-II (250 × 4.6 mm i.d., Nacalai Tesque); solvents, A = 1% acetic