Antioxidant Mechanism Studies on Ferulic Acid: Isolation and Structure Identification of the Main Antioxidation Product from Methyl Ferulate

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Received January 19, 2006; Accepted May 2, 2006

As a part of our research project on the elucidation of the chain-breaking antioxidant mechanism of natural phenolic compounds in food components, ferulic acid, a phenolic acid widely distributed in edible plants, especially grains, was investigated. An antioxidation reaction of ferulic acid methyl ester produced a main product when ethyl linoleate was used as the oxidation substrate. Isolation and structure determination of the main product revealed that it was a dimer having a dihydrobenzofuran moiety. On the basis of the formation pathway for the product, a radical scavenging reaction was suggested to occur between the 5’-position of one of the ferulate radicals and the 2-position of another of the ferulate radicals to terminate the radical chain oxidation of linoleate.

Keywords: ferulic acid; antioxidant reaction, radical scavenging, methyl ferulate dimer

Introduction

Some phenolic compounds in edible plants have received considerable attention as powerful antioxidants that protect against the oxidative deterioration of food. The main mechanism for phenolic antioxidation in food is the trapping and stabilizing of radical species, such as the lipid peroxyl radical, which is generated by radical chain oxidation of food components. The antioxidation process is thought to involve two stages as shown in the following schemes: (Frankel, 1998)

1) radical trapping stage
   S-OO· + AH → S-OOH + A·

2) radical termination stage
   A· → non-radical materials

Where S is the substance that is oxidized, S-OO· is the peroxy radical, AH is the antioxidant, and A· is the antioxidant radical.

Recent mechanistic studies of plant phenolic antioxidant have focused on the trapping stage by adopting a kinetic approach (for ferulic acid, Pan et al., 1999) or a structure-activity relationship approach (for ferulic acid, Nenadis et al., 2003). It should be noted that the antioxidant radical that is produced in the trapping stage must be converted to a non-radical material in the termination stage without the production of any further radical species. Structural information about these non-radical products would represent important contributions to antioxidation mechanism studies (Masuda et al., 1999, 2001, 2002).

Ferulic acid is one of the antioxidatively active phenolic acids (Ou and Kwok, 2004), and is widely distributed in the plant kingdom. The content of ferulic acid in grains is very high. For example, in wheat its level is 50–500 μg/g plant material (Graf, 1992). Ferulic acid occurs mainly in various ester forms with polar compounds such as sugars (Saulnier and Thibault, 1999) and non-polar ones such as sterols in plants (Nystrom et al., 2005). These ester forms of ferulic acid should act as potent antioxidants in plants and also in plant-derived foods. Previously, we investigated the structural effect of the ester part of ferulate on antioxidant efficiency using systematically synthesized esters (Masuda, 2000). The results indicated that shorter alkyl ester had stronger antioxidant activity. In this investigation, we employed methyl ferulate (Fig. 1), the shortest alkyl ester of ferulic acid, as a model of antioxidative ferulic acid esters of edible plants, and clarified the radical scavenging antioxidant mechanism of ferulic esters based on the structure of the antioxidation product from the ferulate.

Materials and Method

Chemicals and instruments 2,2’-Azobis (isobutyronitrile) (AIBN) was obtained from Tokyo Kasei (Tokyo, Japan). Ethyl linoleate was obtained from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel (silica 60, Merck, Darmstat, Germany) chromatography developed using 2.5% ethyl acetate in hexane. All solvents and other reagents were obtained from Nacalai Tesque (Kyoto, Japan). The NMR spectra were measured using a Unity Plus 500 spectrometer (Varian, Palo Alto, CA, USA) or an EX-400 spectrometer (JEOL, Tokyo, Japan) using the manufacturer-supplied pulse sequences [¹H, ¹³C, correlated spectroscopy (HH-COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC)]. The mass...
spectra were measured using an SX-102A spectrometer (JEOL, Tokyo, Japan) in the positive FAB mode in the presence of NaI in a glycerol matrix. A PU-980 high pressure gradient system (JASCO, Tokyo, Japan) equipped with a photodiode array detector (SPD-M10AVP, Shimadzu, Kyoto, Japan) was used for analytical HPLC. A PU-980 pump equipped with a UV-975 detector (JASCO, Tokyo, Japan) was also used for analysis of lipid hydroperoxides. Silica gel column chromatography for sample purification was performed using silica gel BW-300 (Fuji Silysia Chemical, Kasugai, Japan).

Preparation of methyl ferulate To the solution of ferulic acid (20 g) in methanol (400 mL) was added H2SO4 (4 mM) and the mixture was heated at 50°C for 3h. The mixture was poured into chloroform (500 mL) and washed with water (400 mL x 2). The chloroform layer was washed again with a saturated aqueous solution of NaHCO3 (400 mL). The obtained chloroform layer was dried over anhydrous Na2SO4 and evaporated. The residue was purified by silica gel column chromatography and developed with hexane-ethyl acetate (3:1) to obtain methyl ferulate (21 g) as a colorless viscous oil. The obtained ferulate was stored in a freezer (-30°C) until use. 1H NMR (400 MHz, CDCl3) δ ppm 3.80 (3H, s, OCH3), 3.92 (3H, s, OCH3), 5.85 (1H, brs, OH), 6.28 (1H, d, J = 15.0 Hz, H-2), 6.93 (1H, d, J = 8.0 Hz, H-5), 7.03 (1H, d, J = 2.0 Hz, H-2'), 7.08 (1H, dd, J = 2.0 and 8.0 Hz, H-6'), 7.62 (1H, d, J = 15.0 Hz, H-3), HR-EIMS m/z 208.0737 [M]+, calculated for C11H12O4, 208.0736.

Analysis of antioxidant reaction products from methyl ferulate To 35 μL of ethyl linoleate in a 10-mL screw-capped tube were successively added appropriate concentrations of methyl ferulate in acetonitrile solution (1.97 mM) and AIBN (200 mg) to make a reaction solution. After being well stirred by a Vortex (Scientific Industry, Bohemia, NY, USA), the solution was incubated at 40°C with shaking (100 min-1 speed) in the dark by a water bath shaker 11SD (Taitec, Koshigaya, Japan). Twenty-microliter aliquots were taken from the solution at 1-h intervals and diluted with 60 μL of ethyl acetate. Ten microliters of the diluted solution were injected into the HPLC to analyze the antioxidation reaction products from methyl ferulate using the following gradient conditions: column, Cosmosil 55L-II (150 x 4.6 mm i.d.) (Nacalai, Kyoto, Japan); flow rate 1.0 mL/min; gradient mode, from 15% ethyl acetate in hexane (0 min) to 50% ethyl acetate in hexane (25 min), then 100% ethyl acetate for additional 10 min. detection, 330 nm. The concentration of each compound was calculated from the peak area using calibration curves determined for the pure compounds. [Methyl ferulate, Y = 7.69 x 10^5 X - 5.47 x 10^4 (range for X, 12.0–40 mmol) and Y = 7.22 x 10^5 X - 1.89 x 10^4 (range for X, 0.41–12 nmol), Product 1, Y = 9.97 x 10^5 X - 3.66 x 10^4 (range for X, 0.02–10 nmol), where Y is the observed peak area and X is the concentration of each compound.]

Antioxidant activity measurement of methyl ferulate From the antioxidation reaction solution described above, a 10-μL aliquot was taken at 1-h intervals and diluted with 380 μL of methanol. Ten microliters of the diluted solution were injected into the HPLC to analyze the ethyl linoleate hydroperoxides, which were observed at a retention time of 4.9 min under the following conditions: column, YMC-ODS-A (150 x 4.6 mm i.d.) (YMC, Tokyo, Japan), solvent, CH3CN/H2O (9:1), flow rate, 1.0 mL/min, detection, 234 nm. The concentration of hydroperoxides was calculated using the calibration curve: Y = 2.29 x 10^-6 X - 4.38 x 10^-4 (range for X, 9.8 x 10^-2–4.8 x 10^1), where X is the peak area and Y is the concentration of the hydroperoxides (mM).

Isolation of procedure for product 1 To a solution of methyl ferulate (900 mg) in CH3CN (86 mL) was added AIBN (2.2 g). The mixture was heated at 60°C for 5 hrs in the dark. After concentration of the reaction mixture, the residue was subjected to the silica gel column chromatography developed with hexane-ethyl acetate (7:3) to give product 1 (111 mg). The obtained 1 was crystallized by treatment with ether and hexane to give a pale yellow powder. 1, mp 148–152.5°C, HR-FABMS (glycerin + Na) m/z 437.1201 [M + Na]⁺, calculated for C20H16O6Na, 437.1212, 1H NMR (500 MHz, CDCl3) δ ppm 7.63 (1H, d, J = 16.0 Hz, H-5’), 7.33 (1H, brd, J = 1.3 Hz, H-2’), 7.29 (1H, brd, J = 1.3 Hz, H-6’), 7.09 (1H, d, J = 2.0 Hz, H-2’), 6.92 (1H, dd, J = 8.0 and 2.0 Hz, H-6’), 6.84 (1H, d, J = 8.0 Hz, H-5’), 6.44 (1H, d, J = 16.0 Hz, H-5’), 6.03 (1H, d, J = 8.0 Hz, H-3), 4.47 (1H, d, J = 8.0 Hz, H-2), 3.92 (3H, s, 3O-CH3), 3.84 (3H, s, 3O-CH3), 3.81 (3H, s, O-CH3), 3.73 (3H, s, 1O-CH3), 13C NMR (125 MHz, CDCl3) δ ppm 171.6 (C-1), 167.7 (C-1’), 151.0 (C-4’), 148.5 (C-3’), 145.8 (C-3”), 145.5 (C-3’”), 131.9 (C-1’), 129.4 (C-1”), 127.3 (C-5’”), 120.2 (C-6’), 118.9 (C-6”), 116.2 (C-2”), 115.8 (C-5”), 113.4 (C-2’”), 110.7 (C-2’), 88.3 (C-3), 56.4 (3O-CH3), 56.3 (3O-CH3), 55.9 (C-2’), 51.6 (1O-CH3), 53.0 (1O-CH3). Assignments of 1H and 13C signals of 1 were based on the HH-COSY, HSQC, and HMBC of 1.

Results and Discussion
Antioxidant reaction of methyl ferulate The antioxidant reaction conditions were designed by using freshly purified ethyl linoleate (50 mM) as the oxidation substrate and AIBN (0.61 M) as the radical oxidation initiator. The
antioxidant activities of four concentrations (2.4 mM, 4.6 mM, 8.6 mM and 17.0 mM) of methyl ferulate are shown in Fig. 2. Figure 2 shows that methyl ferulate inhibited the accumulation of the hydroperoxide of ethyl linoleate for 5 hr concentration-dependently. Figure 3 shows the HPLC analysis of the reaction mixture from 17.0 mM of methyl ferulate at 5 hr. New peaks at retention times of 13 min (peak I), 14 min (peak II) and 15 min (peak III) are observed besides the large peak of methyl ferulate. Time course analysis of peaks I, II, III, and methyl ferulate peak was carried out using various concentrations of methyl ferulate, these data being shown in Fig. 4. The concentration of methyl ferulate decreased continuously during the antioxidation reaction, whereas peaks I-III increased. While peak II increased continuously during the reaction, peaks I and III decreased after 2 hr under lower concentration conditions (initial ferulate concentrations: 2.4 mM and 8.6 mM), which indicated that products corresponding peaks I and III were not very stable compounds. From these results, peak II should be a typical peak of a stable radical termination product of the antioxidation reaction of methyl ferulate.

Isolation and structure identification of antioxidation product To reveal the structure of the product corresponding to peak II (product 1), I was purified by a chromatographic technique from the reaction mixture as described in the materials and method section. I was isolated as a pale yellow powder. Its molecular formula was determined by high-resolution FAB-MS to be C_22 H_39O_2, which indicated that I was a dimeric compound of methyl ferulate. The ^1H and ^13C NMR data of 1, assignment of the observed peaks in which was carried out by combination of 2D NMRs (HH-COSY, HSQC, and HMBC) as shown in Fig. 5, were very similar to the reported data of one of the benzofuran lignans (Maeda et al., 1994). Therefore, 1 was determined to have structure 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-5-((1E)-3-methoxy-3-oxo-1-propenyl)-3-benzofuran carboxylic acid methyl ester) shown in Fig. 1. Although isolation of the substances corresponding to peaks I and III was also attempted, the isolation yield from each peak was very low (less than one tenth of that of product 1) and both peak substances were found to be a mixture by NMR analysis. Therefore, these substances were estimated to be minor products and further investigation of these substances was not carried out in the present study.

Proposed antioxidant mechanism of methyl ferulate

The antioxidant mechanism for methyl ferulate was considered based on the chemical structure of the main prod-

![Fig. 2. Antioxidant activity of various concentrations of methyl ferulate against AIBN-induced linoleate oxidation (AIBN, 0.61 M, ethyl linoleate, 50 mM).](image1)

![Fig. 3. HPLC analysis of antioxidation reaction (5 h) of methyl ferulate (17 mM).](image2)
Methyl ferulate has one phenolic hydroxyl group, which undergoes hydrogen donation to a lipid peroxyl radical (Shahidi and Wanasundara, 2006) to produce a methyl ferulate radical. Next, two ferulate radicals were coupled between the 2- and 3-position (corresponding to the 2- and 3-positions of one of the aromatic part of one of the ferulate radicals and the 2-position (corresponding to the 2-position of 1) of the alkyl part of another ferulate radical. The produced coupling compound 3 is not very stable because its two aromatic moieties have an unstable quinoid structures. Thus, subsequent addition of an enolated hydroxyl group at the 4-position (corresponding to the 2-position of 1) of one of the aromatic ring occurred at the 3-position (corresponding to the 3-position of 1) of the alkyl part of the other ferulate moieties to form a dihydrobenzofuran structure as shown in structure 1 (Fig. 6). The radical oxidation reaction of the antioxidant has been studied by several researchers (Hall III and Cuppett, 1997). A famous antioxidant, α-tocopherol, afforded quinone and spiro dimer as stable oxidation products (Kawabata et al., 2002). BHT (3,5-di-tert-butyl-4-hydroxytoluene) was reported to be oxidized to yellowing dimers (Zhang et al., 1997). Protocatechuic acid and related phenols gave dimers through their quinone derivatives, which were formed by radical oxidation (Kawabata et al., 2002). Curcumin also gave several dimers, which had a similar dihydrofuran moiety to product 1 (Masuda et al., 1999). Radical oxidation of phenolic antioxidant seemed to give dimeric derivative, however, it depended on the reaction conditions used. Therefore, the importance of dimer for-
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Fig. 6. Proposed radical scavenging antioxidant mechanism of methyl ferulate.
(ROOH and RO· denote lipid hydroperoxide and lipid peroxyl radical, respectively. Tentative position numberings are given on the basis of the numbering system used in 1).

Acknowledgements This study was supported in part by a grant from the Elizabeth Arnold Fuji Foundation (Nagoya, Japan). We thank the Central Instrument Center of the Faculty of Pharmaceutical Science of University of Tokushima for the MS measurement and the Cooperative Center of the University of Tokushima for opportunities to record 1D NMR spectra.

References