Some polyphenols inhibit the formation of pentyl radical and octanoic acid radical in the reaction mixture of linoleic acid hydroperoxide with ferrous ions

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Effects of some polyphenols and their related compounds (chlorogenic acid, caffeic acid, quinic acid, ferulic acid, gallic acid, D-(+)-catechin, D-(–)-catechin, 4-hydroxy-3-methoxybenzoic acid, salicylic acid, L-dopa, dopamine, L-adrenaline, L-noradrenaline, *o*-dihydroxybenzene, *m*-dihydroxybenzene, and *p*-dihydroxybenzene) on the formation of 13-hydroperoxide octadecadienoic (13-HPODE) acid-derived radicals (pentyl radical and octanoic acid radical) were examined. The ESR spin trapping showed that chlorogenic acid, caffeic acid, gallic acid, D-(+)-catechin, D-(–)-catechin, L-dopa, dopamine, L-adrenaline, L-noradrenaline, and *o*-dihydroxybenzene inhibited the overall formation of 13-HPODE acid-derived radicals in the reaction mixture of 13-HPODE with ferrous ions. The ESR peak heights of α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (4-POBN)/13-HPODE-derived radical adducts decreased to $46 \pm 4\%$ (chlorogenic acid), $54 \pm 2\%$ (caffeic acid), $49 \pm 2\%$ (gallic acid), $55 \pm 1\%$ [D-(+)-catechin], $60 \pm 3\%$ [D-(–)-catechin], $42 \pm 1\%$

(L-dopa), $30 \pm 2\%$ (dopamine), $49 \pm 2\%$ (L-adrenaline), $24 \pm 2\%$ (L-noradrenaline), and $54 \pm 5\%$ (*o*-dihydroxybenzene) of the control, respectively. The high performance liquid chromatography–electron spin resonance (HPLC–ESR) and high performance liquid chromatography–electron spin resonance–mass spectrometry (HPLC–ESR–MS) showed that caffeic acid inhibited the formation of octanoic acid radical and pentyl radical to $42 \pm 2\%$ and $52 \pm 7\%$ of the control, respectively. On the other hand, the polyphenols and their related compounds had few inhibitory effects on the radical formation in the presence of EDTA. Visible absorbance measurement revealed that all the polyphenols exhibiting the inhibitory effect chelate ferrous ions. Above results indicated that the chelation of ferrous ion is essential to the inhibitory effects of the polyphenols.

Key words: catechin, chlorogenic acid, free radicals, HPLC–ESR–MS, lipid peroxidation.

INTRODUCTION

Linoleic acid hydroperoxide and hydroperoxides of other unsaturated fatty acids are extremely toxic when injected into mice [1]. Subcutaneous and intravenous injection of linoleic acid peroxide resulted in a marked lesion in intima of the aorta [2,3]. Lipid peroxide-derived radicals seem to be related to the toxicity of the lipid peroxides. Indeed, lipid-derived free radicals, which form in the reaction of lipid peroxides with transition metals are known to cause damage to biomembranes, proteins and the other biomolecules [4–6]. Products analysis and ESR spin-trapping investigations provided evidence for alkoxyl radical intermediates from fatty acid hydroperoxides [7–9]. Furthermore, pentyl radical and 12,13-epoxylinoleic acid radical, which form through β -scission and intramolecular epoxidation of the fatty acid alkoxyl radicals, were detected [10–14].

Chlorogenic acid is an ester of caffeic acid (CA) with quinic acid. It is found naturally in various agricultural products such as coffee beans, potatoes, apples and tobacco leaves. Measurements showed chlorogenic acid to be present in significant quantities: 3.4–14 mg/100 g fresh weight in several varieties of potatoes [15], 12–31 mg/100 ml of juice produced from apples [16], 89 mg/100 g of fresh mature apples [17], 559–674 mg/100 g of dry tea shoots [18]. Catechol derivatives such as caffeic acid are also present at about 250 mg per cup of coffee [19]. Catechin, epicatechin and epicatechin gallate are abundantly contained in green tea and grape seeds. Epicatechin was also detected in red wine and grape juice [20–23]. In addition, catechins and CA are absorbed into the blood stream [24–26]. Thus, it is of interest to

examine the influence of polyphenols such as CA, chlorogenic acid, catechin, on human health in view of their widespread occurrence in food products and the relatively large quantities consumed by virtually the entire human population.

There are many reports showing protective effects of the polyphenols against oxidative stresses. Chlorogenic acid and CA have been known to be inhibitors of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons [27], of retinoic acid 5,6-epoxidation [28], of hydroxyl radical formation [29], and of lipid peroxidation [30,31]. Chlorogenic acid and CA also act as scavengers of superoxide radical, hydroxyl radical [32] and peroxy radical [33]. On the other hand, catechins exert protective effects against oxidative damage of erythrocyte membrane [34], cardiovascular diseases [35,36], inflammatory [37] and cancer [38]. Liu and Mori reported that monoamine metabolites, i.e. norepinephrine and dopamine provide an antioxidant defence in the brain against oxidant- and free radical-induced damage [39]. Neuromelanin also showed a distinct protective effect on lipid peroxidation induced by ferrous ions or water-soluble free-radical initiator, 2,2'-azobis(amidinopropane) dihydrochloride [40]. These protective effects seem to be mainly attributed to their antioxidative activities by preventing the formation of free radicals [41].

However, little is known about the effects of the polyphenols on the formation of respective lipid-derived free radicals. In this study, two kinds of lipid-derived free radicals, i.e. pentyl radical and octanoic acid radical, which form in the reaction of 13-hydroperoxide octadecadienoic acid (13-HPODE) with ferrous ions are separated and identified using the high performance

Abbreviations used: 4-POBN, α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron; CA, caffeic acid; 13-HPODE, 13-hydroperoxide octadecadienoic acid.

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liquid chromatography–electron spin resonance–mass spectrometry (HPLC–ESR–MS) [42]. Furthermore, effects of some polyphenols on the formation of the respective lipid-derived free radicals are examined.

MATERIALS AND METHODS

Materials

Linoleic acid (9,12-octadecadienoic acid), α -(4-pyridyl-1-oxide)-*N*-tert-butyl nitron (4-POBN), D-(–)-catechin, and soybean lipoxygenase (EC 1.13.11.12) Type V were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chlorogenic acid, caffeic acid (CA), quinic acid, ferulic acid, gallic acid, D-(+)-catechin, 4-hydroxy-3-methoxybenzoic acid, L-dopa, dopamine hydrochloride, L-adrenaline, L-noradrenaline, *o*-dihydroxybenzene, *m*-dihydroxybenzene and *p*-dihydroxybenzene were purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). Salicylic acid was from Katayama Chemical Co. (Osaka, Japan). Ferrous ammonium sulphate was obtained from Kishida Chem. Co. (Osaka, Japan). Etylenediaminetetraacetic acid disodium salt (EDTA) was obtained from Wako Pure Chemistries, Ltd. (Osaka, Japan). Pentylhydrazine oxalate was synthesized according to the method of Gever and Hayes [43]. All other chemicals used were of analytical grade.

Preparation of 13-hydroperoxide octadecadienoic acid (13-HPODE)

The reaction mixture contained, in total volume of 25 ml, 1.5 mg/ml linoleic acid, 440 units/ml soybean lipoxygenase, and 0.2 M boric acid (pH 9.0). Reaction was performed at 25 °C under air for 1 h. After 1 h reaction, 0.4 ml of the reaction mixture was mixed with 3.6 ml of 0.2 M borate buffer (pH 9.0), and then injected onto an HPLC–UV system. The HPLC–UV used consisted of a model 7125 Rheodyne injector (Reodyne, Cotati, CA, U.S.A.), a model Hitachi 655A-11 pump with a model L-5000 LC controller (Hitachi Ltd., Ibaragi, Japan), a Water μ Bondapak C₁₈ semipreparative column (Millipore Co., Milford, MA, U.S.A.) (30 mm $\times$ 10 mm I.D.), and a model SPD-M10AVP diode array detector (Shimadzu Co., Kyoto, Japan) with a model CLASS-LC10 LC workstation (Shimadzu Co., Kyoto, Japan). The SPD-M10AVP diode array detector was operated at 200–350 nm in the HPLC–UV system. Two solvents were used in the HPLC–UV: A, water; B, methanol. A combination of isocratic and linear gradient was used for the HPLC–UV: 0–20 min, 0–90 % B (linear gradient) at flow rate 2.0 ml/min; 20–30 min, 90 % B (isocratic) at flow rate 2.0 ml/min. A prominent peak was observed at a retention time of 20.3 min when the HPLC profile was monitored at 235 nm. The peak fraction was collected. The methanol contained in the fraction was removed using a model CC-105 centrifugal concentrator with a model TU-055 Low Temperature Trap (Tomy Ltd., Yokohama, Japan). The water solution of the fraction was used as a stock solution of 13-HPODE [44]. The concentration of 13-HPODE was determined from its absorbance at 234 nm ($\epsilon = 25\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$) [45].

Control reaction mixture of 13-HPODE with ferrous ions

The control reaction mixture of 13-HPODE with ferrous ions contained 140 μM 13-HPODE, 0.33 mM $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$, 0.1 M 4-POBN, and 38 mM phosphate buffer (pH 7.4). The reaction was started by adding $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$. The reaction was performed for 2 min at 25 °C.

Figure 1 ESR spectra of the reaction of 13-HPODE with ferrous ions

The reaction and ESR conditions were as described in the Materials and methods section. **A**, Control reaction mixture; **B**, control reaction mixture without ferrous ions; **C**, control reaction mixture without 13-HPODE.

ESR measurements

The ESR spectra were obtained using a model JES-FR30 Free Radical Monitor (Jeol Ltd., Tokyo, Japan). Aqueous samples were aspirated into a Teflon tube centred in a microwave cavity. Operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.1 mT; centre of magnetic field, 337.200 mT; sweep time, 4 min; sweep width, 10 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO ($\Delta H_{3-4} = 8.69\text{ mT}$).

Visible absorption spectra

Visible absorption spectra were measured using a model UV-160A ultraviolet-visible spectrophotometer (Shimadzu Co., Kyoto, Japan). The spectrophotometer was operated from 400 nm to 800 nm. The measurements were performed at 25 °C. In the reference cell, 38 mM phosphate buffer (pH 7.4) was contained. Sample solutions consisted of 38 mM phosphate buffer (pH 7.4), 1 mM chlorogenic acid (or CA, or quinic acid, or ferulic acid, or gallic acid, or D-(+)-catechin, or D-(–)-catechin, or 4-hydroxy-3-methoxybenzoic acid, or salicylic acid, or L-dopa, or dopamine, or L-adrenaline, or L-noradrenaline, or *o*-dihydroxybenzene, or *m*-dihydroxybenzene, or *p*-dihydroxybenzene), and 0.33 mM $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$.

HPLC–ESR analysis

The HPLC used in the HPLC–ESR consisted of a model 7125 injector (Reodyne, Cotati, CA, U.S.A.) with a 5 ml sample loop, a model 655A-11 pump with a model L-5000 LC controller (Hitachi Ltd., Ibaragi, Japan). A Water μ Bondapak C₁₈ semipreparative column (30 mm $\times$ 10 mm I.D.) (Millipore Co., Milford, MA, U.S.A.) was used. The column was kept at 25 °C throughout the analyses. For the HPLC–ESR analyses, two solvents were used: solvent A, 50 mM ammonium acetate; solvent B, 50 mM ammonium acetate/acetonitrile (20:80, v/v). A combination of isocratic and linear gradient was used: 0–30 min, 100 % A to 20 % A (linear gradient) at flow rate 2.0 ml/min; 30–40 min, 20 % A (isocratic) at flow rate 2.0 ml/min. The eluent was introduced into a model JES-FR30 Free

Figure 2 ESR spectra of the reaction of 13-HPODE with ferrous ions in the presence of CA

The reaction and ESR conditions were as described in the Materials and methods section. Amplitude of the ESR spectra **D** and **E** was half of the ESR spectra **A**, **B**, and **C**. **A**, Control reaction mixture. **B**, 1 mM CA was added to the control reaction mixture at 0 min. **C**, 1 mM CA was added to the control reaction mixture at 1.5 min. **D**, Control reaction mixture with 1 mM EDTA. **E**, CA was added to the control reaction mixture with 1 mM EDTA at 0 min.

Radical Monitor (Jeol Ltd., Tokyo, Japan). The ESR spectrometer was connected to the HPLC with a Teflon tube, which passed through the centre of the ESR cavity. The operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.2 mT; time constant, 1 s. The magnetic field was fixed at the third ESR peak indicated by arrow (Figure 1) throughout the experiments.

Synthesis of pentyl radical

Authentic pentyl radical was synthesized through the decomposition of pentylhydrazine. The reaction mixture contained, in total volume of 1 ml, 0.1 M 4-POBN, 2.5 mg pentylhydrazine oxalate, 0.2 mM CuCl_2 , and 45 mM carbonate buffer (pH 10.0). After nitrogen gas was bubbled through the reaction mixture without CuCl_2 for 5 min, the reaction was started by adding CuCl_2 . Reaction was performed for 2 h at 25 °C. 10 μl of the pentyl radical reaction mixture was mixed with 1.49 ml of 50 mM phosphate buffer (pH 7.4) and then applied to the HPLC-ESR.

Figure 3 Time course of 4-POBN/13-HPODE radical adduct formation

The reaction and ESR conditions were as described in the Materials and methods section. (○), Control reaction mixture. (●), 1 mM CA was added to the control reaction mixture at 0 min. (□), 1 mM CA was added to the control reaction mixture at 1.5 min. (△), The control reaction mixtures with 1 mM EDTA. (▲), 1 mM CA was added to the control reaction mixture with 1 mM EDTA at 0 min.

HPLC-ESR-MS analysis of the peak 1 and peak 2 radical adducts

The HPLC-ESR-MS consisted of a model 7125 injector (Reodyne Cotati, CA, U.S.A.), a model L-7100 pump (Hitachi Ltd., Ibaragi, Japan), a Water μ Bondapak C_{18} semi-preparative column (30 mm $\times$ 10 mm I.D.) (Millipore Co., Milford, MA, U.S.A.), a model JES-FR30 Free Radical Monitor (Jeol Ltd., Tokyo, Japan), and a model M-1200AP LC-MS system with an electrospray ionization (ESI) (Hitachi Ltd., Ibaragi, Japan). The HPLC and ESR conditions in the HPLC-ESR-MS analysis were same as those in the HPLC-ESR analysis except for the HPLC mobile phase. For the HPLC-ESR-MS analyses, two solvents were used: solvent A, 50 mM acetic acid; solvent B, 50 mM acetic acid/acetonitrile (20:80, v/v). A combination of isocratic and linear gradient was used: 0–30 min, 100% A to 20% A (linear gradient) at flow rate 2.0 ml/min; 30–40 min, 20% A (isocratic) at flow rate 2.0 ml/min. The operating conditions of the mass spectrometer were: nebulizer, 180 °C; aperture 1, 120 °C; N_2 controller pressure, 2.0 kgf/cm²; drift voltage, 70 V; multiplier voltage, 1800 V; needle voltage, 3000 V; polarity, positive; resolution, 48.

Figure 4 Structures of polyphenols and their related compounds

The reaction mixture contained, in total volume of 1.5 ml, 140 μ M 13-HPODE, 0.33 mM $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$, 0.1 M 4-POBN, 1 mM EDTA and 38 mM phosphate buffer (pH 7.4). The reaction was started by adding $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$. After 2 min reaction at 25 $^\circ\text{C}$, the reaction mixture was applied to the HPLC-ESR. Peaks from three HPLC-ESR chromatograms were collected and combined. After the volume of the combined sample had been reduced to about 1 ml, the HPLC-ESR-MS analysis was performed. The mass spectra of the peaks 1 and 2 were obtained by introducing the eluent from the ESR detector into the LC-MS system just before the respective peaks were

eluted. The flow rate was kept at 50 μ l/min while the eluent was introducing into the LC-MS system.

RESULTS

ESR measurements of the reaction mixtures of 13-HPODE with ferrous ions

ESR spectra of the control reaction mixture, the control reaction mixture without 13-HPODE, and the control reaction mixture without ferrous ions were measured (Figure 1). An ESR spectrum ($a^N = 1.58$ mT and $a^H\beta = 0.26$ mT) was observed in the control

Figure 5 Effects of polyphenols and their related compounds on the formation of 13-HPODE-derived radicals

The ESR spectra were observed for the control reaction mixture with 1 mM of chlorogenic acid (or CA, or quinic acid, or ferulic acid, or gallic acid, or D-(+)-catechin, or D-(-)-catechin, or 4-hydroxy-3-methoxybenzoic acid, or salicylic acid, or L-dopa, or dopamine, or L-adrenaline, or L-noradrenaline, or *o*-dihydroxybenzene, or *m*-dihydroxybenzene, or *p*-dihydroxybenzene). The reaction was started by adding $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$. The reaction was performed for 2 min at 25 °C. Signal intensities were evaluated from the peak height of the third ESR signal of the 4-POBN/13-HPODE-derived radical adducts. The control value 100% represents the level of 4-POBN/13-HPODE-derived radical adducts formed in the absence of the compounds. The respective values are means $\pm$ SD of five determinations. ESR conditions were as described in the Materials and methods section.

reaction mixture (Figure 1A). ESR peaks were hardly observed in the absence of ferrous ions (or 13-HPODE) (Figures 1B and 1C).

Effects of CA on the formation of the 13-HPODE-derived radicals

In order to examine the effects of CA on the overall formation of 13-HPODE-derived radicals, ESR spectrum of the control reaction mixture with 1.0 mM CA was measured (Figure 2B). On addition of CA to the control reaction mixture, ESR peak height decreased to $54 \pm 2\%$ of the control. To check whether or not CA inhibits the formation of 13-HPODE-derived radicals or degrades the radical adducts of 4-POBN with 13-HPODE-derived radicals, CA was added into the control reaction mixture at 1.5 min after the reaction was started. Addition of CA at 1.5 min resulted in no effects on the ESR peak height, indicating that CA inhibits 13-HPODE-derived radicals formation itself (Figure 2C). To know whether or not chelation of Fe^{2+} ions is essential to the inhibitory effects, EDTA was added to the control reaction mixture (Figure 2D). On addition of EDTA to the control reaction mixture, the ESR peak height increased to $270 \pm 30\%$ of the control, indicating that EDTA/ferrous ion complex is more reactive than phosphate ion/ferrous ion complex in the reaction mixture. Addition of CA into the control reaction mixture with EDTA resulted in little effect on the ESR peak height ($96 \pm 6\%$) (Figure 2E). These results indicated that CA inhibits the formation of 13-HPODE-derived radicals through the chelation of iron ions.

Figure 6 HPLC-ESR analysis of the reaction of 13-HPODE with ferrous ions

The reaction and HPLC-ESR conditions were as described in the Materials and methods section. **A**, 1.5 ml of control reaction mixture; **B**, 1.5 ml of control reaction mixture with 1 mM CA; **C**, 1.5 ml of control reaction mixture with 1 mM EDTA; **D**, 1.5 ml of control reaction mixture with 1 mM EDTA and 1 mM CA.

Time course experiments

Time course experiments of the ESR peak height were performed (Figure 3). For the control reaction mixture, the reaction reached plateau at 2 min after the reaction was started. When CA was added into the control reaction mixture at 0 min, smaller ESR peaks were observed compared with the control during time course experiment. The reaction also reached a plateau in 2 min. The ESR peak heights of the 4-POBN/13-HPODE-derived radical adducts remained unchanged for the control reaction mixture with CA during a further 28 min incubation, indicating that the ESR spin adducts are stable in the reaction mixture. When CA was added into the control reaction mixture at 1.5 min, the ESR peak height was almost the same as the control. The ESR peak height also remained unchanged during a further 28.5 min incubation. CA apparently does not convert the 4-POBN/13-HPODE-derived radical adducts to ESR silent species. The above results indicated that CA inhibits 13-HPODE-derived radical formation itself.

Time course experiment was performed for the control reaction mixture with EDTA in the presence of CA (or in the absence of CA). The control reaction mixture with EDTA reached a plateau in 7 min. On the other hand, when CA was added into the control reaction mixture with EDTA, the ESR peak height continued to increase during a 28 min incubation.

Effects of some polyphenols and their related compounds on the formation of the 13-HPODE-derived radicals

Effects of some polyphenols and their related compounds (Figure 4) on the overall formation of the 4-POBN/13-HPODE-derived

Figure 7 The HPLC-ESR analysis of control reaction mixture and the reaction mixture of authentic 4-POBN/pentyl radical adduct

The reaction and HPLC-ESR conditions were as described in the Materials and methods section. **A**, 1.5 ml of control reaction mixture; **B**, 10 μ l of reaction mixture of authentic 4-POBN/pentyl radical adduct was mixed with 1.49 ml of 50 mM phosphate buffer (pH 7.4) and applied to the HPLC-ESR; **C**, 1.49 ml of control reaction mixture was mixed with 10 μ l of reaction mixture of authentic 4-POBN/pentyl radical adduct and applied to the HPLC-ESR.

Figure 8 HPLC-ESR-MS analysis of the peak 1 and peak 2 fractions

The reaction and HPLC-ESR-MS conditions are as described in the Materials and methods section. **A**, peak 1; **B**, peak 2.

radical adducts were examined (Figure 5). The ESR peak heights of the 4-POBN/13-HPODE-derived radical adducts decreased to $46 \pm 4\%$ (chlorogenic acid), $49 \pm 2\%$ (gallic acid), $55 \pm 1\%$ [D-

Figure 9 Effect of oxygen concentration on the formation of peak 1 and peak 2 radical adducts

The HPLC-ESR conditions are as described in the Materials and methods section. After nitrogen gas was bubbled into the control reaction mixture without $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$ for 5 min, the reaction was started by adding $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4$. The reaction was performed for 2 min at 25 $^\circ\text{C}$. 1.5 ml of the reaction mixture was applied to the HPLC-ESR analysis. **A**, control reaction mixture under air; **B**, control reaction mixture under nitrogen gas.

(+)-catechin], $60 \pm 3\%$ [D-(+)-catechin], $42 \pm 1\%$ (L-dopa), $30 \pm 2\%$ (dopamine), $49 \pm 2\%$ (L-adrenaline), $24 \pm 2\%$ (L-noradrenaline), and $54 \pm 5\%$ (*o*-dihydroxybenzene) of the control, respectively. On the other hand, quinic acid ($99 \pm 4\%$), ferulic acid ($104 \pm 5\%$), 4-hydroxy-3-methoxybenzoic acid ($93 \pm 4\%$), salicylic acid ($91 \pm 5\%$), *m*-dihydroxybenzene ($107 \pm 5\%$), and *p*-dihydroxybenzene ($94 \pm 4\%$) showed no effects on the overall formation of the 4-POBN/13-HPODE-derived radical adducts.

Visible absorption spectra of the mixtures of ferrous ions with some polyphenols and related compounds

Visible absorption spectra were measured for the mixtures of Fe^{2+} ions with chlorogenic acid (or other polyphenols and related compounds). Of the polyphenols and their related compounds, chlorogenic acid, CA, gallic acid, D-(+)-catechin, D-(+)-catechin, L-dopa, dopamine, L-adrenaline, L-noradrenaline, and *o*-dihydroxybenzene showed characteristic absorbance bands around 550 nm. The absorbance bands were not observed for the mixtures without ferrous ions (or the polyphenols), indicating the formation of polyphenol chelates of Fe^{2+} ions. The λ_{max} (nm) and absorbances of the polyphenol chelates of Fe^{2+} ions are as follows: chlorogenic acid [488 nm (1.37) and 640 nm (1.12)], CA [591 nm (0.859)], gallic acid [552 nm (0.617)], D-(+)-catechin [559 nm (0.584)], D-(+)-catechin [562 nm (0.630)], L-dopa [559 nm (0.552)], dopamine [546 nm (0.496)], L-adrenaline [534 nm (0.768)], L-noradrenaline [532 nm (0.773)], and *o*-dihydroxybenzene [562 nm (0.292)].

The HPLC-ESR analysis of the control reaction mixture, the control reaction mixture with CA, the control reaction mixture with EDTA, and the control reaction mixture with EDTA and CA

In order to know the effect of CA on the formation of respective 13-HPODE-derived radicals, the HPLC-ESR analyses were performed for the control reaction mixture, the control reaction mixture with CA, the control reaction mixture with EDTA, and the control reaction mixture with EDTA and CA. On the HPLC-ESR elution profile of the control reaction mixture, two

Scheme 1 Proposed mechanism for the formation of pentyl radical and octanoic acid radical in the reaction of 13-HPODE with ferrous ions

prominent peaks were observed at the retention times of 22.1 ± 0.3 min (peak 1) and 31.3 ± 0.3 min (peak 2), respectively (Figure 6A). When CA was added to the control reaction mixture, respective peak heights decreased to $42 \pm 2\%$ (peak 1) and $52 \pm 7\%$ (peak 2) of the control (Figure 6B), indicating that CA inhibits the formation of the peak 1 and peak 2 compounds, respectively. On the other hand, on addition of EDTA to the control reaction mixture, respective peak heights increased to $150 \pm 20\%$ (peak 1) and $170 \pm 30\%$ (peak 2) of the control (Figure 6C). In the presence of EDTA, addition of CA to the control reaction mixture resulted in little effect on the formation of the peak 1 ($90 \pm 8\%$) compound and peak 2 compound ($89 \pm 8\%$) (Figure 6D).

In order to identify the peak 1 and peak 2 compounds, 10 μ l of authentic pentyl radical reaction mixture was mixed with

1.49 ml of 50 mM phosphate buffer (pH 7.4) and then the HPLC–ESR analysis was performed for the solution (Figure 7B). The 4-POBN/pentyl radical adduct was eluted at the retention time of 31.4 min. The retention time is identical with peak 2 compound. When 1.5 ml of the control reaction mixture was mixed with 10 μ l of authentic pentyl radical reaction mixture and then applied to the HPLC–ESR, peak height of the peak 2 compound increased (Figure 7C). Thus, peak 2 compound was shown to be 4-POBN/pentyl radical adduct.

The HPLC-ESR-MS analysis of the control reaction mixture with EDTA

To identify the radical species formed in the control reaction mixture with EDTA, the HPLC-ESR-MS experiments were

performed [48]. Mass spectra of peak 1 and peak 2 fractions are shown in Figure 8.

The HPLC-ESR-MS analysis of peak 1 compound gave ions at m/z 251, m/z 338 and m/z 675, respectively. The ion m/z 338 corresponds to the protonated molecular ion of 4-POBN/octanoic acid radical adduct, $(M+H)^+$. A fragment ion at m/z 251 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion. The ion m/z 675 is the protonated dimer ion, $(2M+H)^+$.

The HPLC-ESR-MS analysis of peak 2 compound gave ions at m/z 179, m/z 266 and m/z 531, respectively. The ion m/z 266 corresponds to the protonated molecular ion of 4-POBN/pentyl radical adduct, $(M+H)^+$. A fragment ion at m/z 179 corresponds to the loss of $[(CH_3)_3C(O)N]$ from the protonated molecular ion. The ion m/z 531 is the protonated dimer ion, $(2M+H)^+$.

Effects of oxygen concentration on the formation of peak 1 and peak 2 radical adducts

Effects of oxygen concentration on the formation of peak 1 and peak 2 radical adducts were examined using the HPLC-ESR (Figure 9). Peak 1 radical adduct was predominant under air. On the other hand, peak 2 radical adduct was predominant under nitrogen gas.

DISCUSSION

In this study, 4-POBN/octanoic acid radical adduct and 4-POBN/pentyl radical adduct were detected and identified in the reaction mixture of 13-HPODE with ferrous ions using the HPLC-ESR-MS. Possible reaction paths for the formation of these radicals are shown in Scheme 1. Our previous studies have shown the formation of octanoic acid radical and pentyl radical in the reaction mixture of linoleic acid with soybean lipoxygenase [12,14]. Product analysis and spin-trapping studies provided evidence for the formation of 13-alkoxylinoleic acid radical through the reaction of 13-HPODE with ferrous ions [7-9]. The β scission of 13-alkoxylinoleic acid radical results in the formation of pentyl radical [11-14]. The pentyl radical could be a precursor of pentane. Garssen et al. [46] have reported the formation of pentane and 13-oxo-9,11-tridecadienoic acid in the reaction mixture of soybean lipoxygenase with linoleic acid. On the other hand, 13-alkoxylinoleic acid radical possibly rearranges intramolecularly by addition to a double bond to cause the formation of 12,13-epoxylinoleic acid radical. Indeed, 12,13-epoxylinoleic acid radical was detected and identified in the reaction mixture of soybean lipoxygenase with linoleic acid in borate buffer (pH 9.0) [14]. In this study, however, 4-POBN/12,13-epoxylinoleic acid radical adduct was not detected by the HPLC-ESR-MS. It may be due to the different reaction conditions. Phosphate buffer (pH 7.4) was employed in this study. On the other hand, 4-POBN/12,13-epoxylinoleic acid radical adduct was detected in borate buffer (pH 9.0) [14]. The reaction of 12,13-epoxylinoleic acid radical with oxygen molecule results in the formation of 12, 13-epoxy-9-hydroperoxylinoleic acid radical. Gardner et al. [47] have reported that 12,13-epoxy-9-hydroperoxylinoleic acid forms in the reaction mixture of 13-HPODE with cysteine-FeCl₃. Octanoic acid radical (peak 1) possibly forms through β scission of 12,13-epoxy-9-alkoxylinoleic acid radical. The reaction between 13-alkoxylinoleic acid radical and 12,13-epoxylinoleic acid radical seems to be reversible [48] (Scheme 1) because the HPLC-ESR-MS peak height of 4-POBN/pentyl radical adduct increased under nitrogen gas (Figure 9).

Polyphenols possibly inhibit the following two steps, i.e. step 1, the reaction between 13-HPODE and 13-alkoxylinoleic acid radical, and step 2, the reaction between 12,13-epoxy-9-hydro-

peroxylinoleic acid and 12,13-epoxy-9-alkoxylinoleic acid radical because ferrous ions participate in the two reactions. Formation of octanoic acid radical is inhibited at both steps in the reaction paths. On the other hand, formation of pentyl radical is inhibited only at step 1. The HPLC-ESR peak heights decreased to $42 \pm 2\%$ (octanoic acid radical) and $52 \pm 7\%$ (pentyl radical) of the control. Formation of octanoic acid radical was more effectively inhibited. The measurement is consistent with the above reaction paths. The HPLC-ESR-MS analysis allowed us to examine effects of the polyphenols on the respective radical formations.

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