酸化防止剤と金属の複合反応による活性酸素種の産生

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Original Paper

Generation of Reactive Oxygen Species by Interaction between Antioxidants Used as Food Additive and Metal Ions

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Food additives, such as preservatives, sweeteners, coloring agents, and flavoring agents, are widely used in food manufacturing. However, their combined effects on the human body are not known. The purpose of this study was to examine whether combinations of antioxidants and metal ions generate reactive oxygen species (ROS) under *in vitro* conditions using electron spin resonance (ESR). Among the metal ions examined, only iron and copper generated ROS in the presence of antioxidants. Moreover, certain phenolic antioxidants having pro-oxidant activity induced DNA oxidation and degradation *via* the generation of high levels of ROS in the presence of copper ion, resulting in complete degradation of DNA *in vitro*.

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Key words: antioxidant; food additive; metal ion; oxidative stress; reactive oxygen species

Introduction

Food additives, such as preservatives, sweeteners, coloring agents, and flavoring agents, are commonly used in food manufacturing. The use of food additives is regulated by the Ministry of Health, Labour and Welfare in Japan, based on doses determined not to adversely affect human health by the Food Safety Commission.

Food additives are classified as specified additives and existing additives. Existing additives are additives that have been used for a long time in Japan. However, madder color, a food coloring extracted from the roots of *Rubia tinctorum* L., has been deleted from the list of existing additives because it was reported to induce renal carcinogenesis[1, 2] following a re-evaluation of food additives. The European Food Safety Authority (EFSA) has initiated an internal mandate for preparing refined exposure calculations for food colors re-evaluated since 2010[*1, *2].

The FAO/WHO Joint Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) determine the acceptable daily intake (ADI) based on all the facts available at the time of evaluation. JECFA generally sets ADI values based on the lowest relevant no-observed-adverse-effect level (NOAEL) in the most sensitive test species. The ADI values for food additives are commonly derived from NOAEL values in long-term *in vivo* studies in animals. To derive an ADI value, a safety or uncertainty factor (commonly 100) is applied to the NOAEL in the most sensitive test species. However, unexpected effects caused by combined reactions may arise, because the ADI is determined for single compounds. There are many components in food and drink; thus, it is quite likely that interactions occur in the human body.

In our previous study, we found that phenolic compounds with antioxidant activity react with metal ions (particularly copper ion), generating reactive oxygen species (ROS)[3]. Caffeic acid (CaA), a phenolic compound, reacted with sodium nitrite in artificial gastric juice, forming new products *via* generation of reactive nitrogen species (RNS)[4].

Antioxidants are added to many foods to prevent the degradation of food components and degradation or fading of food colors. Generally speaking, antioxidants scavenge ROS and inhibit ROS-induced oxidative DNA damage. However, phenolic compounds are redox agents and can act as pro-oxidants under certain conditions. It seems likely that this is also the case for other antioxidants.

The purpose of this study was to examine the interaction of antioxidants with metal ions *in vitro*, using electron spin resonance (ESR), in order to determine whether these is a potential risk to human health.

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*1 Commission Regulation (EU) No. 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No. 1333/2008 of the European Parliament and of the Council on food additives.

*2 Food additive re-evaluations given fresh impetus with new call for data, 27 March 2013, http://www.efsa.europa.eu/en/press/news/130327.htm

Materials and Methods

1. Chemicals and reagents

The chemical structures of the antioxidants used in this study are shown in Fig. 1. L-Ascorbic acid (Asc) was obtained from Kanto Chemical (Tokyo, Japan). 3,4-Dihydroxycinnamic acid (caffeic acid; CaA), *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid; FA), gallic acid (GA), glycyrrhizin (Gly), γ-oryzanol (Ory), quercetin dihydrate (Que), rutin (Rut), γ-tocopherol (Toc), and 2,6-di-*t*-butyl-4-methylphenol (butylhydroxytoluene; BHT) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Carnosic acid (Car), (+)-catechin (Cat), chlorogenic acid (ChA), eugenol (Eug), phytic acid (PA), sesamin (Semin), sesamol (Semol), and 4-hydroxy-3-*tert*-butylanisole (BHA) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Sodium chloride (Na^{+}), magnesium chloride (Mg^{2+}), potassium chloride (K^{+}), calcium chloride (Ca^{2+}), manganese(II) acetate tetrahydrate (Mn^{2+}), manganese(IV) oxide (Mn^{4+}), iron(III) chloride (Fe^{3+}), copper(I) chloride (Cu^{+}), and zinc chloride (Zn^{2+}) were purchased from Wako Pure Chemical Industries. Chromium(III) chloride hexahydrate (Cr^{3+}), ammonium iron(II) sulfate hexahydrate (Fe^{2+}) and copper(II) sulfate pentahydrate (Cu^{2+}) were obtained from Kanto Chemical.

α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) as a spin-trapping reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Tokyo Chemical Industry. Dimethyl sulfoxide (DMSO), hydrogen peroxide (30%), and nuclease P1 were obtained from Wako Pure Chemical Industries. Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, disodium salt (EDTA) and bathocuproinedisulfonic acid disodium salt (BCS) were obtained from Dojindo Laboratories (Kumamoto, Japan). Catalase (CAT), deferoxamine mesylate salt (DFO), deoxyribonucleic acid sodium salt from calf thymus, phosphatase alkaline from bovine intestinal mucosa, 2′-deoxyguanosine (dG), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were obtained from Sigma (Tokyo, Japan). 2′-Deoxyguanosine-$^{13}C_1$,$^{15}N_2$ (dG-$^{13}C_1$,$^{15}N_2$) and 8-hydroxy-2′-deoxyguanosine-$^{13}C_1$,$^{15}N_2$ (8-OHdG-$^{13}C_1$,$^{15}N_2$) used as an internal standard (IS) were purchased from Toronto Research Chemicals (Toronto, USA).

Ultrapure water was provided by a Milli-Q integral 3 system. Other chemicals and solvents were obtained from Wako Pure Chemical Industries.

2. DPPH radical-scavenging activity

The modified DPPH method was used for determination of antioxidant activity[3, 4]. DPPH radical solution (0.1 mmol/L) was prepared in methanol and the antioxidants were diluted with methanol to concentrations ranging from 0.1 to 2 mmol/L. In a 1.5 mL disposable tube, the prepared DPPH (200 µL) solution was added to a sample of diluted antioxidant (200 µL). The mixed

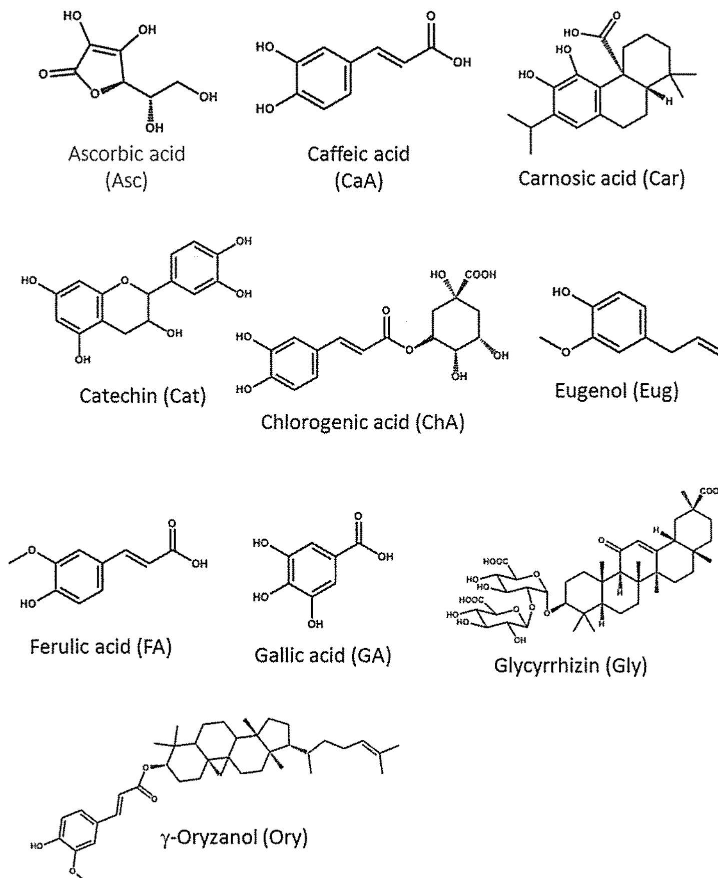

Fig. 1. Chemical structures of antioxidants examined

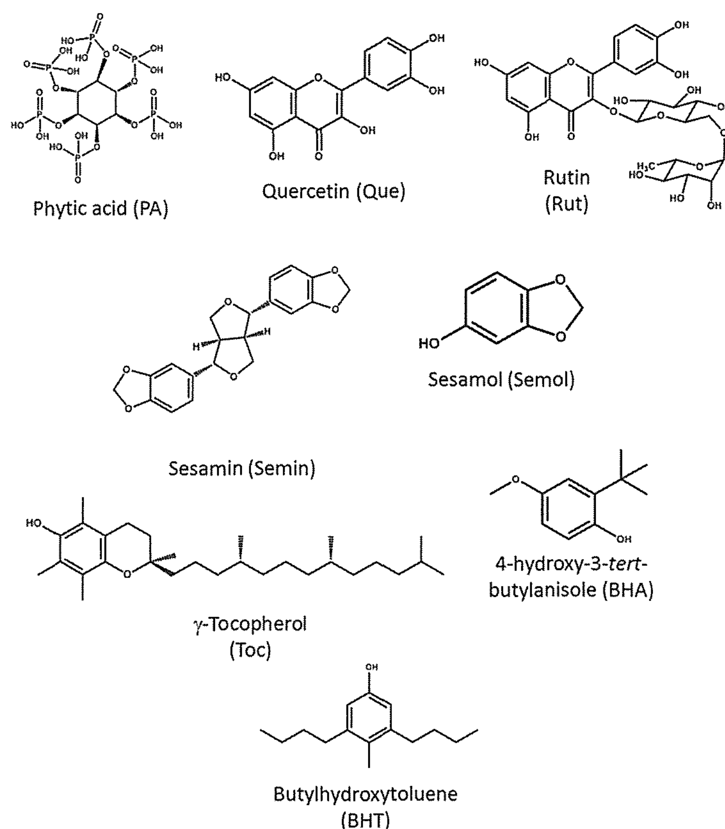

Fig. 1 Continued

Table 1. MRM conditions for 2′-deoxyguanosine and 8-hydroxy-2′-deoxyguanosine

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
2′-Deoxyguanosine	268.1	152.0	25	11
2′-Deoxyguanosine-$^{13}C_1$,$^{15}N_2$	271.1	155.0	25	11
8-Hydroxy-2′-deoxyguanosine	283.9	167.9	17	13
8-Hydroxy-2′-deoxyguanosine-$^{13}C_1$,$^{15}N_2$	286.9	170.9	17	13

samples were incubated for 30 min at 40℃. Then, the absorbance was measured at 540 nm with a BIO-RAD iMark™ microplate reader. The effect of antioxidants on DPPH absorbance was estimated. DPPH scavenging activity was determined according to the following equation: % scavenging activity=[Abs. (control)−Abs. (sample)]/[Abs. (control)−Abs. (ascorbic acid)]×100. DPPH plus ascorbic acid (10 mmol/L) was used as a positive control. The values of the effective concentration (concentration able to inhibit 50% of the oxidation (EC_{50})) were calculated from the inhibition percentage.

Table 2. Antioxidant activities of phenolic compounds as assessed by DPPH assay

Antioxidant compound	EC_{50} (μmol/L)	Antioxidant compound	EC_{50} (μmol/L)
Asc	109	Ory	196
CaA	106	PA	2,295
Ca	96	Que	49
Cat	64	Rut	57
ChA	111	Semin	N.A.
Eug	82	Semol	135
FA	133	Toc	129
GA	37	BHA	89
Gly	N.A.	BHT	128

Final DPPH concentration was 50 μmol/L. N.A.: no activity

3. Electron spin resonance measurement of hydroxyl radical

The ESR method was used for the determination of hydroxyl radicals (˙OH)[3, 4]. Instead of directly trapping ˙OH, this method utilizes POBN to trap methyl radical (˙CH_3) that is formed by the interaction of DMSO with ˙OH, and measures the trapped ˙CH_3. Moreover, this method can detect ˙OH, which is not affected by copper ion. The analysis of ˙CH_3 was carried out with an ESR spectrometer (JES-RE1X, JEOL Co., Tokyo, Japan). The ESR spectrum was measured at a microwave frequency of 9.43 GHz, a magnetic field of 336.0±5 mT, a microwave power of 9.0 mW, a modulation of 100 kHz, a time constant of 0.03 s, and a sweep time of 30 s, using the ESR spectrometer. The spectra of the samples were scanned to record the signal intensities (peak-to-peak heights).

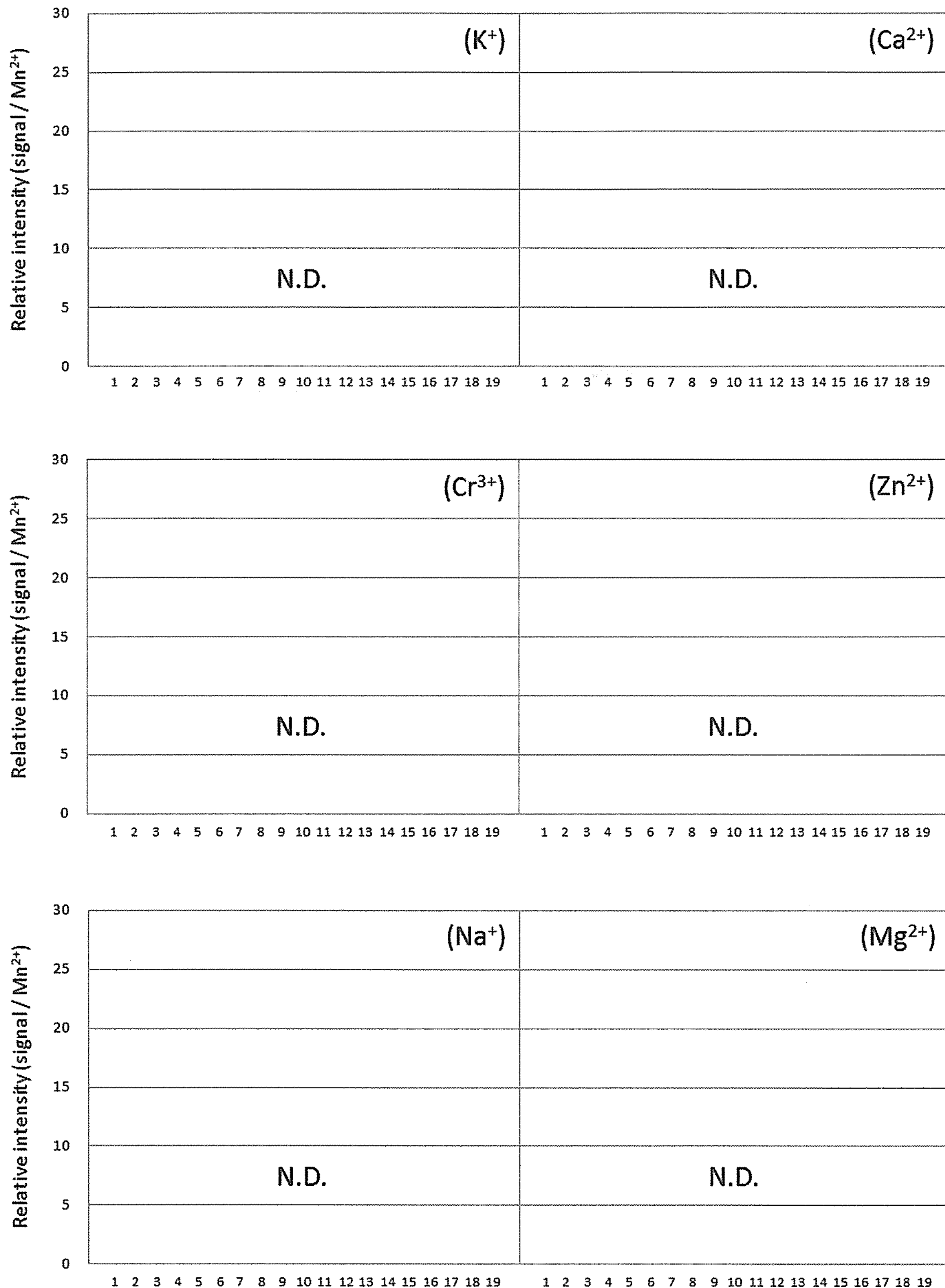

Fig. 2. Effect of various metal ions on pro-oxidant activities

Data are means±S.D. ($n=3$).
N.D.: not detected.
The sample solution (0.3 mL) containing phosphate-buffered saline (pH 7.4), POBN (10 mmol/L), DMSO (10%), antioxidant (1 mmol/L), and metal ion (1 mmol/L) was incubated at 40°C for 30 min, and then the ESR spectrum was measured under the conditions as described in Materials and Methods.
1. Control (methanol); 2. Ascorbic acid; 3. Caffeic acid; 4. Carnosic acid; 5. Catechin; 6. Chlorogenic acid; 7. Eugenol; 8. Ferulic acid; 9. Gallic acid; 10. Glycyrrhizin; 11. γ-Oryzanol; 12. Phytic acid; 13. Quercetin; 14. Rutin; 15. Sesamin; 16. Sesamol; 17. γ-Tocopherol; 18. 4-Hydroxy-3-*tert*-butylanisole; 19. Butylhydroxytoluene

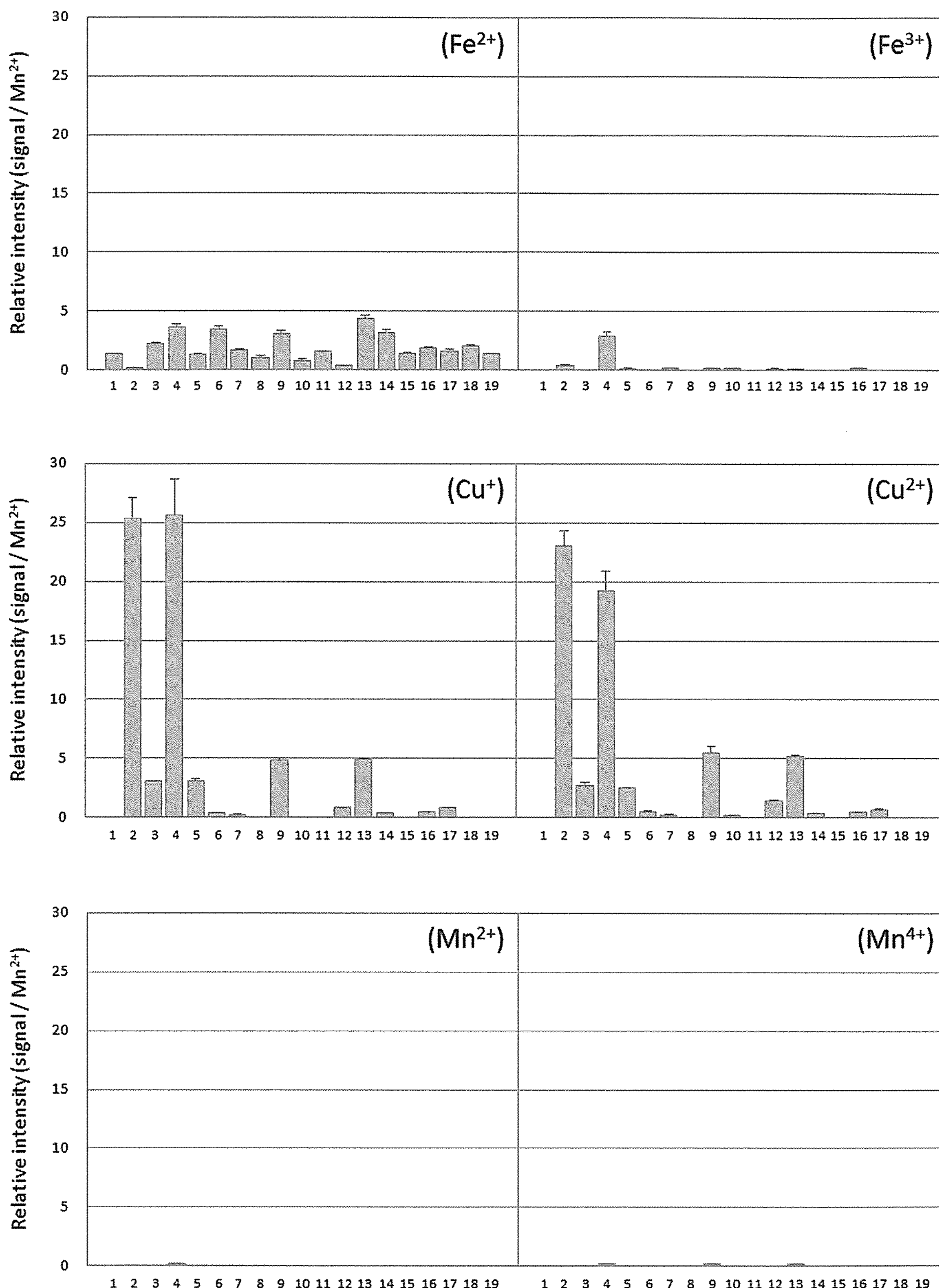

Fig. 2. Continued

A typical reaction mixture for incubation at 40℃ for 30 min consisted of phosphate-buffered saline (pH 7.4), POBN (10 mmol/L), DMSO (10%), antioxidant (1 mmol/L), metal ion (1 mmol/L), and hydrogen peroxide (10 mmol/L) in a final volume of 0.3 mL.

4. DNA digestion and determination of dG and 8-OHdG

In order to prevent the formation of oxidative by-products during DNA isolation, DNA was digested by using a slightly modification of our previous method[3, 4]. Calf thymus DNA (2 mg/mL, 0.4 mL) was incubated at 40℃ for 30 min after addition of 0.05 mL of antioxidant

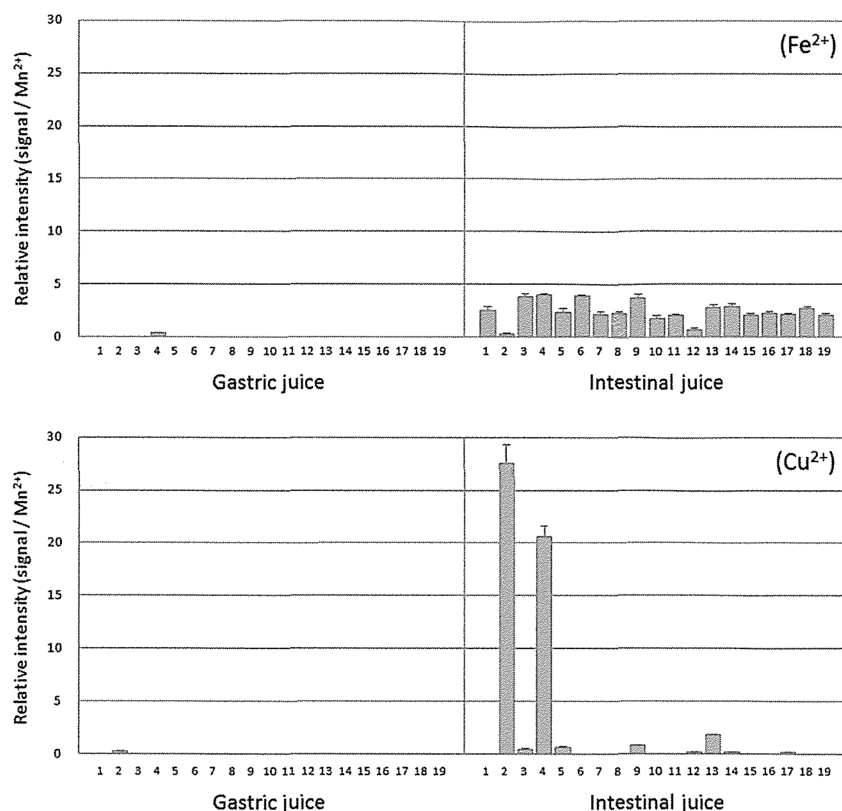

Fig. 3. Comparison of pro-oxidant activities in artificial gastric and intestinal juice

Data are means±S.D. ($n = 3$).
The sample solution (0.3 mL) containing artificial gastric or intestinal juice, POBN (10 mmol/L), DMSO (10%), antioxidant (1 mmol/L), and metal ion (1 mmol/L) was incubated at 40°C for 30 min, and then the ESR spectrum was measured under the conditions as described in Materials and Methods.
1. Control (methanol); 2. Ascorbic acid; 3. Caffeic acid; 4. Carnosic acid; 5. Catechin; 6. Chlorogenic acid; 7. Eugenol; 8. Ferulic acid; 9. Gallic acid; 10. Glycyrrhizin; 11. γ-Oryzanol; 12. Phytic acid; 13. Quercetin; 14. Rutin; 15. Sesamin; 16. Sesamol; 17. γ-Tocopherol; 18. 4-Hydroxy-3-*tert*-butylanisole; 19. Butylhydroxytoluene

(1 mmol/L), 0.05 mL of Cu^{2+} (1 mmol/L), 0.05 mL of hydrogen peroxide (10 mmol/L), and 0.45 mL of phosphate-buffered saline (pH 7.4). The treated calf thymus DNA was immediately centrifuged at 10,000×g for 5 min at 10℃ after addition of 0.3 mL of NaI (7.6 mol/L) and 0.5 mL of 2-propanol. The pellet was washed with 40% 2-propanol and 70% ethanol, and then dissolved in 0.20 mL of sodium acetate buffer (20 mmol/L, pH 4.8) before adding 10 μL of internal standard (2 mmol/L dG-$^{13}C_1$,$^{15}N_2$ and 20 μmol/L 8-OHdG-$^{13}C_1$,$^{15}N_2$). DNA was enzymatically hydrolyzed by adding 5.0 μL of nuclease P1 (500 units/mL). The mixture was incubated at 60℃ for 15 min. After the addition of 20 μL of 1.0 mol/L Tris–HCl buffer (pH 8.0), 5.0 μL of alkaline phosphatase (1,000 units/mL) was added. The mixture was incubated at 40℃ for 60 min and then passed through a 3,000 NMWL filter (Millipore, Tokyo, Japan). The digested solution was injected into the column-switching LC-MS/MS instrument for 8-OHdG and dG analysis.

LC-MS/MS analyses were performed using an Alliance HPLC system (Waters, Japan). On-line solid-phase extraction was accomplished on a Shodex ODP-50 4B column (50 mm×4.6 mm, 5 μm; Showa Denko, Japan) and analyte separation was achieved on a Shiseido Capcell Pak C18 MGII column (250 mm×2.0 mm, 5 μm; Shiseido, Japan). Column temperature was maintained at 40℃ and the flow rate was set to 0.2 mL/min. The sample extract was injected onto the precolumn, which was flushed with 20 mmol/L acetate buffer (pH 4.7) at a flow rate of 0.3 mL/min. Mobile phases for separation were (A) water, (B) methanol, and (C) 100 mmol/L acetic acid. A gradient program was used as follows: 0–9 min, 5% B and 5% C; 9–10 min, 5–90% B and 5% C; 10–14.5 min, 90% B and 5% C; 14.5–15 min, 90–5% B and 5% C; 15–20 min, 5% B and 5% C. Autosampler temperature was set to 4℃ and 30 μL was injected.

MS/MS detection was performed with a Micromass Quattro micro API triple quadrupole mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operating in the positive mode. The optimization results for the two most abundant ion transitions of analytes in the multiple reaction monitoring (MRM) mode are given in Table 1. The following optimum conditions were set: source temperature 120℃ and desolvation temperature 500℃. Cone and desolvation gas flow rates were set at 50 and 450 L/h, respectively.

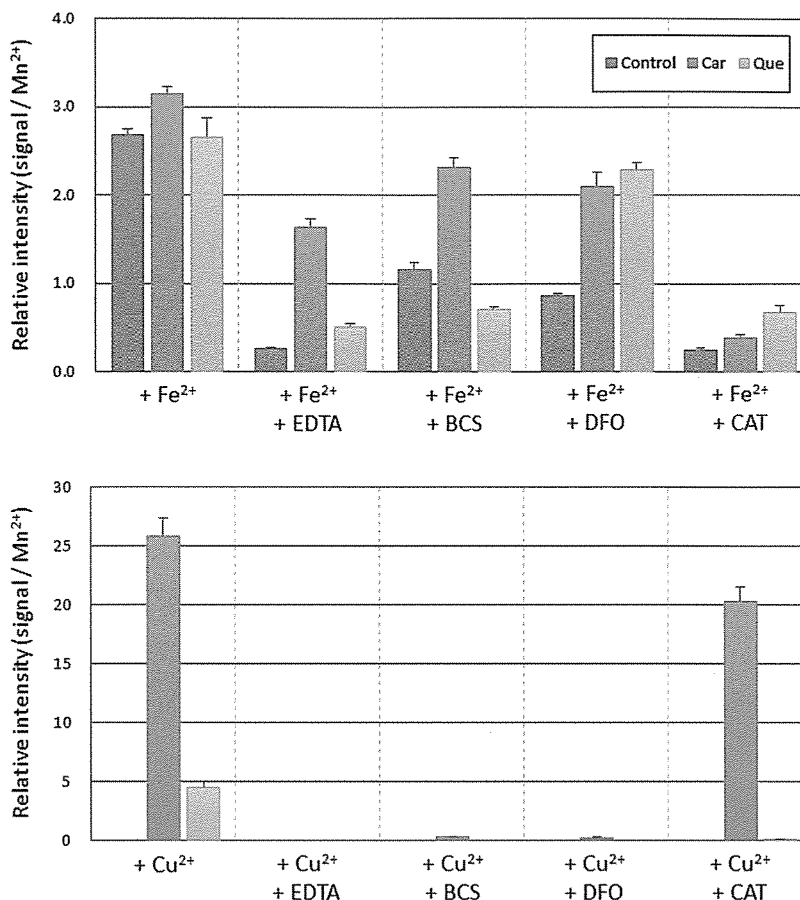

Fig. 4. Effect of chelators and enzymes on ROS generation

Data are means±S.D. ($n = 3$).
The sample solution (0.3 mL) containing phosphate-buffered saline (pH 7.4), POBN (10 mmol/L), DMSO (10%), chelator (10 mmol/L), antioxidant (1 mmol/L), and metal ion (1 mmol/L) was incubated at 40°C for 30 min, and then the ESR spectrum was measured under the conditions as described in Materials and Methods.
Control, methanol; Car, carnosic acid; Que, quercetin; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; BCS, bathocuproinedisulfonic acid; DFO, deferoxamine mesylate; CAT, catalase

5. Agarose gel electrophoresis of DNA

Electrophoresis was performed on 2% agarose gel in the presence of Tris–acetate buffer (40 mmol/L Tris base, 20 mmol/L acetic acid, and 1 mmol/L EDTA; pH 8.0) and 0.5 μg/mL ethidium bromide. Treated DNA samples in sample buffer containing 5% glycerol and 0.001% bromophenol blue were applied to the well. DNA fragments were separated by electrophoresis at 100 V for 1 h and detected with a UV transilluminator.

Results and Discussion

1. Assessment of antioxidant activities of antioxidants used as food additives

Antioxidant activities were measured by the DPPH method, which is a well established method for evaluating antioxidant activity. Freshly prepared DPPH solution has a deep purple color with an absorption maximum at 517 nm. This color generally disappears in the presence of an antioxidant. The scavenging effect of antioxidants on DPPH free radicals is summarized in Table 2. All the antioxidants showed strong antioxidant activities with the exception of Gly, PA, and Semin. Active compounds having more than one hydroxyl group on the aromatic ring, such as Cat, GA, Que, and Rut, tended to have high antioxidant activity.

Antioxidant activity blocks ROS-mediated damage (through radical scavenging) and/or suppresses the generation of ROS (by binding metal ion). Further, DPPH radical-scavenging activity was correlated with hydrogen peroxide-scavenging activity[5]. Dihydroxy substitution of phenolic compounds, *i.e.*, an *ortho*-dihydroxy structure (catechol structure) of the B-ring, which possesses electron-donating properties and is a radical target, is important for antioxidant activity[6].

2. Effect of interaction between antioxidants and metal ions on generation of reactive oxygen species

Minerals and trace elements are necessary for many physiological and biological functions. Iron, the most abundant transition metal in biological tissues, is an essential component of many important metalloproteins. Copper is a component of several metalloenzymes. Zinc

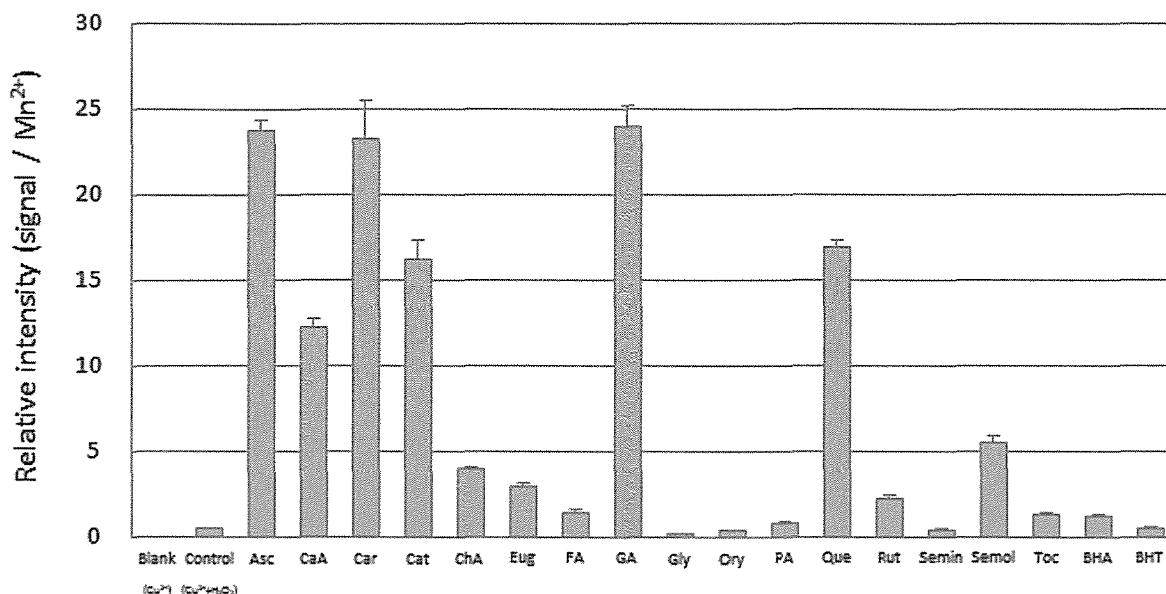

Fig. 5. Pro-oxidant activities of combinations of antioxidants, copper ion, and hydrogen peroxide

Data are means±S.D. ($n=3$).
The sample solution (0.3 mL) containing phosphate-buffered saline (pH 7.4), POBN (10 mmol/L), DMSO (10%), antioxidant (1 mmol/L), copper ion (1 mmol/L), and hydrogen peroxide (10 mmol/L) was incubated at 40°C for 30 min, and then the ESR spectrum was measured under the conditions as described in Materials and Methods.

is implicated in the function of more than 200 enzymes. These minerals and trace elements are found not only in dietary foods but also in food supplements.

There are several methods for free radical determination, including fluorescence[7] and liquid chromatography[8]. However, we selected ESR because it is specific and reliable. We measured ROS generated by the interaction between antioxidants and metal ions by ESR and evaluated the pro-oxidant activities of chromium, manganese, iron, copper, zinc, sodium, magnesium, potassium, and calcium. Among them, only iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{+} and Cu^{2+}) generated ROS by interacting with antioxidants. Copper (Cu^{+} and Cu^{2+}) ions had the highest pro-oxidant activity (Fig. 2).

In our second series of experiments, the antioxidants were reacted with Fe^{2+} or Cu^{2+} in artificial gastric or intestinal juice. Low levels of ROS were generated by almost all antioxidants in the presence of Fe^{2+} in artificial intestinal juice. In the presence of Cu^{2+}, substantial amounts of ROS were generated from Asc and Car, but not from other antioxidants, in artificial intestinal juice (Fig. 3). In gastric juice, no ROS generation was observed. ROS such as superoxide anion and hydroxyl radical are generated by degradation of hydrogen peroxide under alkaline conditions[9].

The effects of chelators and enzymes on ROS generation were next examined. EDTA, bathocuproinedisulfonic acid (BCS; a specific Cu^{+} chelator), deferoxamine mesylate (DFO; a specific Fe^{3+} chelator)[10], and catalase (CAT; H_2O_2 scavenger) decreased ROS generation induced by antioxidants and metal ions (Fig. 4). DFO has a high affinity for Fe^{3+} but a very low affinity for Fe^{2+}[11]. BCS can chelate Cu^{+} after reduction of Cu^{2+} to Cu^{+} *via* electron transfer from antioxidants. Therefore, it seems reasonable to conclude that the pro-oxidant activity of antioxidants depends on the generation of hydrogen peroxide, by certain metal ions.

In general, phenolic compounds induce ROS in the presence of Cu^{2+}. The finding that phenolic compounds showed higher reactivity for copper ions than other metal ions can be explained in terms of the redox potentials. For instance, the standard reduction potential of the Cu^{2+}/Cu^{+} couple (+0.15 V) is much lower than those of the Fe^{3+}/Fe^{2+} couple (+0.77 V) and the Mn^{3+}/Mn^{2+} couple (+1.56 V)[12),13]. On the other hand, the redox potentials of phenolic compounds Que and Cat are +0.40 V and +0.44 V, respectively[14]. Antioxidants can reduce Cu^{2+} to Cu^{+}. However, iron and manganese cannot induce the redox cycle because they require a high redox potential to reduce the metal. Consequently, redox cycling in the presence of an antioxidant, leading to various ROS, occurs only with copper ion.

3. Measurement of hydroxyl radical and oxidative damage in calf thymus DNA

It is well known that copper or iron ions can induce $^{\cdot}OH$ generation through the Fenton reaction in the presence of hydrogen peroxide. Our results indicated that antioxidants have both antioxidant and pro-oxidant activities. Thus, we examined the interactions among antioxidant, hydrogen peroxide, and copper ion by ESR and found that Asc, CaA, Car, Cat, GA, and Que all promoted ROS generation *via* the Fenton reaction (Fig. 5).

Increasing oxidative stress mediated by ROS causes DNA oxidation, which is involved in the pathogenesis of various diseases, such as cancer. Thus, we measured 8-OHdG, a DNA oxidative stress marker, by means of column-switching LC-MS/MS. We found that almost all

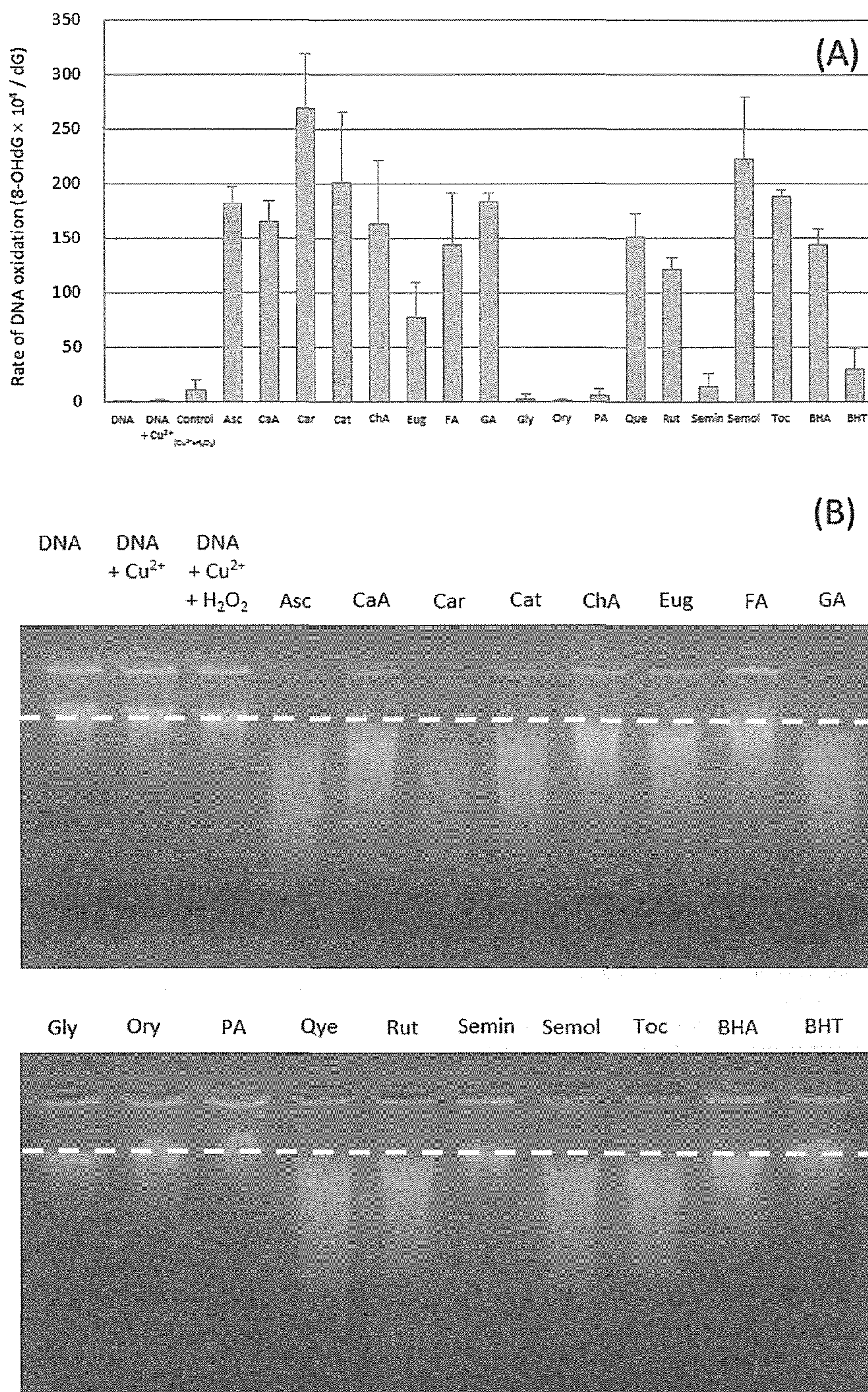

Fig. 6. Analysis of oxidation and degradation of DNA from calf thymus by (A) column-switching LC-MS/MS and (B) agarose gel electrophoresis with UV detection

of the antioxidants examined induced DNA oxidation and degradation, in agreement with the ESR results (Fig. 6). There were some of inconsistencies with the ESR data, but antioxidants can behave both as antioxidants and pro-oxidants, depending on many factors, such as concentration, reaction rate or experimental conditions. It is noteworthy that Asc and Car generated high levels of ROS in the presence of copper ion, and caused complete DNA degradation. Colorectal cancer is associated with both genetic predisposition and inflammation[15], and inflammation is associated with the release of large amounts of ROS and RNS leading to oxidation of nucleic acids.

Our results indicate that combinations of high concen-

trations of antioxidants and certain metal ions can generate large amounts of ROS in artificial intestinal juice. Further studies will be needed to evaluate the physiological significance of these findings.

Conclusion

Our results show that phenolic antioxidants generate ROS in the presence of iron and copper ions. In particular, compounds with *ortho*-dihydroxy groups may chelate Cu^{2+} to generate the highest pro-oxidant activity. The initial electron-transfer oxidation by Cu^{2+} generates the corresponding semiquinone radical, which would undergo a second electron-transfer reaction with O_2 to form *ortho*-quinone and superoxide anion ($O_2^{-\cdot}$). Then $O_2^{-\cdot}$ reacts with Cu^{+} to produce hydrogen peroxide, which is readily converted into $^{\cdot}OH$ *via* a Fenton-like reaction[16]. Thus, certain antioxidants can generate large amounts of ROS in the presence of copper ion, and induce DNA degradation *in vitro*. The physiological significance of this reaction remains to be investigated.

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酸化防止剤と金属の複合反応による活性酸素種の産生（報文，英文）

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食品添加物は，保存料，甘味料，着色料，香料など，食品の製造過程または食品の加工・保存の目的で使用されている．個別では安全とうたわれていた食品中の化学物質でも生体内で相互的な複合反応を引き起こし，想定外な影響を与える可能性がある．本研究では食品添加物の安全性を評価するために，酸化防止剤と金属の複合反応に着目し，ラジカルを選択的に検出可能な電子スピン共鳴装置（ESR）を用いた活性酸素種（ROS）生成の評価を行った．酸化防止剤と各種金属を反応させたところ，鉄と銅以外のミネラルや微量金属はROSの産生に寄与しなかった．しかし，特定の酸化防止剤は銅と反応することで，ROSが産生されDNAの酸化と切断を引き起こし，酸化ストレスを惹起させていることが示唆された．

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