Original Article

Bioavailability of dietary antioxidants in Acanthopanax sieboldianus Makino leaves

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Abstract

We have investigated the intestinal absorption and metabolism of the polyphenols contained in *Acanthopanax sieboldianus* Makino (Ukogi in Japanese) in rat plasma using a liquid chromatography/mass spectrometry (LC/MS). Ukogi mainly contained chlorogenic acid, rutin, and kaempferol 3-*O*-rutinoside. These polyphenols were detected in non-hydrolyzed rat plasma 0.5 hour after oral administration, and the concentration of rutin was 13.6 ± 3.65 nM. The metabolized compounds of kaempferol sulfoglucuronide and 3'-*O*-methylquercetin sulfoglucuronide were identified by exact mass determination and exact mass mass spectrometry/mass spectrometry (MS/MS) in the plasma of rats fed a 5 % Ukogi diet for 8-24 days. Antioxidant potential of the metabolized compounds is discussed concerning a beneficial effect in the body via antioxidation caused by not only scavenging superoxide but also blocking superoxide generation.

Key words

LC/MS, metabolism, MS/MS, polyphenol, Ukogi

1. Introduction

There are many kinds of polyphenol compounds in vegetables, fruits, and medicinal plants. It is thought that these polyphenols work as antioxidants and have the beneficial effect of reducing the risk of certain degenerative diseases such as cancer and cardiovascular diseases. Thus, the impact of several diseases may be ameliorated by simply improving the dietary intake of nutrients with antioxidant properties. Actually, it was reported (DaSilva et al., 1998; Hayek et al., 1997; Salvayre et al., 1992; Miura et al., 1995; Whalley et al., 1990) that the consumption of pure flavonoids or dietary foods containing polyphenols led to the prevention of LDL oxidation by scavenging reactive oxygen species (ROS) and chelating iron, which inhibits the generation of ROS. However, it is unclear whether or not the polyphenols are absorbed and metabolized in the body.

We have paid attention to the leaves of *Acanthopanax sieboldianus* Makino, Ukogi in Japanese. This plant came from China and belongs to the Araliaceae family. It is used as a tonic remedy for the heart and circulatory system in Chinese medicine, and is thought to be a medicine for perpetual youth and longevity in China. In Japan, it has been cultivated as hedges and used for food and medicine since the Heian era, 1300 years ago. Ukogi has high levels of saponins and polyphenols such as kaempferol 3-*O*-rutinoside (Sawada et al., 1993). It is expected to be a high-level antioxidant in dietary foods. The levels of lipid peroxides and total cholesterol in rats following the administration of Ukogi were lower than those of rats on a vitamin E-free diet (Yamada et al., 1997). In addition, improvements in the blood glucose level, glucose tolerance and serum triglyceride following administration of Ukogi were reported by Tabuchi et al. (2003). However, the relationship between the beneficial effects of Ukogi and the antioxidant actions of Ukogi polyphenols is unclear. It is necessary to ascertain the bioavailability of Ukogi polyphenols and to consider their antioxidant activities based on the processes of absorption, elimination, and changes in the molecular structure *in vivo*.

The bioavailability of flavonoid glycosides in biological systems has been studied by the hydrolysis of flavonoid glycosides and their metabolites. Hollmans et al. (1996; 1997) reported that the hydrolysis of quercetin glycosides, quercetin glucuronides, and quercetin sulfates could be conducted using a 2 M HCl aqueous methanol solution. The total concentration of quercetin was determined by using high-performance liquid chromatography (HPLC) with a fluorescence detector. Manach et al. (1996; 1997; 1999), Morand et al. (1998; 2000), and Crespy et al. (2002) reported a method for determining the concentration of deconjugated metabolites with β-glucuronidase and sulfatase using HPLC with an electrochemical detector. On the other hand, liquid chromatography/mass spectrometry (LC/MS) can directly detect flavonoid glycosides or their metabolites in biological systems. Shimoi et al. (1998) detected a negligibly small ion peak of a flavonoid metabolite using a LC/fast atom bombardment mass spectrometry (FAB-MS) method. However, LC/FAB-MS is unsuitable for quantitative analysis of trace amounts of flavonoid glycosides or identification of unknown metabolites because many ions in the matrix interfere with finding the target ions. Recently, it was reported that flavonoids and their metabolites could be measured by LC/electrospray ionization mass spectrometry (ESI-MS). Oliveira et al. (2002) also detected flavonoid glucosides derived from tablets of *Ginko biloba* (total flavonoid glycosides 57.6 mg) in human plasma after 3 hours

on the final day of a 4-day administration using LC/ESI-MS. Mauri et al. (1999) also confirmed the presence of intact rutin in human plasma by LC/ESI-MS after the consumption of tomato puree for two weeks, but could not detect any flavonoid metabolites in the plasma. In addition, the major circulating compounds in the human plasma after 1.5 hr were identified as quercetin-3-glucuronide, 3'-methylquercetin-3-glucuronide and quercetin-3'-sulfate by Day et al. (2001). There appears to be dissension among researchers because the time between feeding and blood collection. In addition, the analyzing equipment and experimental animals used differ. The circulating metabolites and flavonoide glycoside would be changed by the time between feeding and blood collection. It is necessary to analyze unknown metabolites without hydrolyzing the plasma.

The use of LC/ESI/quadrupole orthogonal-acceleration time-of-flight mass spectrometry (Q-oaTOF-MS) enables exact mass determination of unknown metabolites in the plasma. From the exact mass determined in this manner, the elemental composition of an unknown compound can be calculated. In addition, exact mass mass spectrometry and mass spectrometry (MS/MS) allows elemental composition of fragments to be determined. Combination of the retention time data in a specified programmed gradient elution, UV spectra, product ion spectra, calculated possible elemental compositions, and a database should allow the identification of unknown metabolites present in the plasma.

The purpose of our research was to determine the values of the polyphenols and their metabolites in plasma, because absorption and metabolism may drastically modify their antioxidant properties. Two types of studies in rats have been performed: single does study and a long-term multiple does study. In order to identify the metabolites, exact mass and exact mass MS/MS by LC/ESI/Q-oaTOF-MS was used.

2. Materials and methods

2.1 Materials

Chlorogenic acid, quercetin 3-*O*-rutinoside (rutin), kaempferol, acetic acid, and ethanol were purchased from Wako Pure Chemical Co. (Tokyo, Japan). Quercetin was purchased from Aldrich Chemical Company Inc (Milwaukee, USA). Kaempferol 3-*O*-rutinoside, quercetin 3-*O*-arabinoglucoside (Peltatoside), fisetin and 4'5, 7-trihydroxy-3'-methoxyflavonol (isorhamnetin) were purchased from Funakoshi Co. (Tokyo, Japan). The other reagents used were commercially available.

2.2 Quantification of polyphenols in Ukogi

Fresh Ukogi leaves were dried for three minutes using a microwave oven. Ukogi powder was prepared by crushing the dried Ukogi leaves in a blender (Osaka Chemical Co.). Ukogi powder (0.5 g) was extracted three times with 80 % (v/v) ethanol (10 ml). The extract was dried to approximately 20 ml under vacuum by rotary evaporation (30 ℃) and made up to 25 ml with 80 % EtOH. An aliquot (1 ml) was filtered (0.45 μm, PTFE filter units, Whatman Inc., Clifton, USA) and subjected to HPLC-diode array detection (Jasco, Tokyo, Japan). Chlorogenic acid, rutin and kaempferol 3-*O*-rutinoside in Ukogi powder were quantified by reversed-phase HPLC on an C30-UG-5 column (150 × 2.0 mm, 5 μm, Nomura chemical. Co., Ltd., Aich, Japan). Solvents A (water: H_3PO_4, 100:0.1, v:v) and B (acetonitrile: H_3PO_4, 100:0.1, v:v), were run at flow rate of 0.2 ml/min. The separation was performed as follows: 0 min 5 % B; 0-40 min: linear gradient to 25 % B. The peaks were identified by comparison of retention times, UV spectra with those of authentic standards.

2.3 Animal and diets

In single dose experiment, male Wistar rats (9 weeks old, 210-240 g, Clea Japan Inc., Tokyo, Japan) were housed in an air-conditioned room (23 ± 3 ℃) under 12 h dark/12 h light cycles, with free access to tap water and CE-2 (Clea Japan inc., Tokyo, Japan). Four rats were assigned to each experimental group.

Long-term dose for 8 days was conducted with 8 week-old male Wistar rats of about 250 g (Clea Japan, Tokyo, Japan). In addition, the effects of longer dose for 15 days and for 24 days were examined using 7 week-old male Wistar rats with about 200 g. The rats were housed in an air-conditioned room (23 ± 3 ℃) under 12 h dark/12 h light cycles with free access to trap water and a control synthetic basic diet (AIN-93, Clea Japan, Tokyo, Japan) containing : 46.6 % (w/w) corn starch, 14 % (w/w) milk casein, 15.5 % (w/w) α corn starch, 10 % (w/w) sugar, 4 % (w/w) refined soybean oil, 5 % (w/w) cellulose powder, 3.5 % (w/w) minerals, 1 % (w/w) vitamins, 0.18 % (w/w) L-cystine, 0.25 % (w/w) choline ditartrate, and 0.0008 % (w/w) tertiary butyl hydroquinone. For Ukogi meal, the control diet was supplemented with 5 % (v/v) Ukogi powder.

2.4 Sampling procedure

In single dose, rat fasted overnight was administrated Ukogi extract (the mixture of 0.5 g Ukogi powder incubated in 4 ml boiling water) or water by gastric intubation. And then they were anesthetized with pentobarbital sodium. Blood samples were collected from the portal vein into heparinized tubes at 0.5, 1, 2, 4, 6, 8, 12 and 24 h after dosing. Plasma was obtained by centrifugation (4 ℃, 3000 rpm, 10 min). In long-term feeding, rats fasted overnight were anesthetized with pentobarbital sodium, and blood was withdrawn from the abdominal aorta into heparinized tubes. These samples were stored at -80 ℃ until use.

2.5 Direct detection of rutin, chlorogenic acid and conjugated metabolites from plasma

For determination of rutin and conjugated metabolites

the plasma (400 µl) was added to 1.6 ml 1 % acetic acid in ethanol. Quantification of rutin by LC/ESI- selected ion monitoring (SIM) was added to 20 µl of 16.8 µM peltatoside as an internal standard. These samples were vortexed for 1 min and centrifuged (12500 *g*) at 4 °C for 10 min. The ethanol phases were collected and evaporated with a rotary evaporator. Each dried extract was dissolved in 2 ml 1 % (v/v) acetic acid in water, and eluted through a NEXUS cartridge. After washing the cartridge with 1 % (v/v) acetic acid in water, 50 % (v/v) methanol eluates were obtained. The eluates were evaporated to dryness and the residues were dissolved in 200 µl of 20 % (v/v) methanol in water. These were passed through 0.45 µm filters and 20 µl of each solution was injected into the HPLC system.

Conjugated metabolites were detected by HPLC-diode alley detector (DAD). The column (YMC-Pac-Pro C18, 75 × 2.0 mm, 3 µm, YMC Co., Ltd. Japan) was eluted at 35 °C. Solvents A (water: H_3PO_4, 100:0.1, v:v) and B (acetonitrile: H_3PO_4, 100:0.1, v:v),were run at flow rate of 0.2 ml/min. The separation was performed as follows: 0 min 15 % B; 0-10 min: linear gradient to 25 % B and maintained B at 25 % (20 min).

For determination of chlorogenic acid by LC/electrochemical detector (ECD), 170 µl plasma was added to 20 µl of 0.1mM fisetin as an internal standard, 10 µl of H_2O and 800 µl of methanol-H_3PO_4 (100:0.1, v/v). Samples were centrifuged (12500 rpm, 4 °C, 10 min), and passed through 0.45 µm filters and 20 µl of each solution was injected into the HPLC system. ECD was performed with an ESA coulochem II (MC Medical, Japan), and the potentials were set at 100 mV for chlorogenic acid and fisetin (I.S.). Solvent A (50 mM NaH_2PO_4 buffer (pH 3) : methanol , 99:1, v/v) (A) and B (100 mM NaH_2PO_4 buffer (pH 3.45) : acetonitrile : methanol, 3:6:1, v/v/v) , were run at a flow rate of 1 ml/min, using a gradient of 10 % B, increasing to 48 % B (38 min), 55 % B (2 min), and maintained B at 55 % (30 min) and increasing to 99 % B (2 min). The column (C30-UG-5, 250 × 4.6 mm, 5 µm, Nomura chemical. Co., Ltd., Aich, Japan) was eluted at 30 °C. The detector was set to 100mV. The recovery of this method has been checked (> 85 %) using pure chlorogenic acid in plasma and the limit detection was 0.05 µM.

2.6 HPLC/ESI-MS analysis

To detect the polyphenols and their metabolites, two LC/ESI-MS instrument systems were used. The first was a Jasco 980 Galliver series gradient LC system (Tokyo, Japan) with UV detection (280 nm) and a Micromass ZabSpec-Q mass spectrometer equipped with an electrospray interface (Micromass Ltd., Manchester, UK). Mass spectra were collected in the negative electrospray mode and acquired from 100-1100 Da at a spectrum integration time of 3.5 s with an inter-acquisition delay of 1.5 s. The needle voltage, sampling cone, and ring electrode were 8 kV, 4 kV, and 4 kV, respectively. Data acquisition and processing were carried out using OPUS. To confirm the metabolites and components of Ukogi, HPLC condition was adjusted by volatility solvents. The column (C30-UG-5, 150 × 2.0 mm, 5 µm) was eluted at 35 °C. Solvents A (water: CH_3COOH, 100:1, v/v) and B (acetonitrile: CH_3OOH, 100:1, v/v) were run at flow rate of 0.2 ml/min using a linear gradient from 10 to 30 % B for 20 min. Quantitative measurements of rutin and kaempferol 3-*O*-rutinoside were accomplished by SIM at m/z 609.1415 $[M-H]^-$ and m/z 593.1506 $[M-H]^-$, respectively. Peltatoside was selected as an internal standard at m/z 595.1299. The conditions were a scan window of 0.36, dwell times, 0.1 s/channel and an interchannel delay of cycle time of 0.02 s. A linear curve was obtained from 5-50 nM. The recovery of this method has been checked using pure rutin in plasma and the limit detection was 5 nM in plasma. HPLC isocratic conditions were $H_2O/CH_3CN/CH_3COOH$, 82:18:1 (v/v/v). The sample (20 µl) was injected into the HPLC, and the flow rate was 0.2 ml/min.

The second LC/ESI-MS system was a Waters 2690 Separation Module (Waters Co., MA, USA) with a 486 tunable absorbance detector (Waters Co., MA, USA) and a Micromass Q-oaTOF mass spectrometer equipped with a two-way ESI source (LC/ESI/Q-oaTOF-MS) (Micromass Ltd., Manchester, UK). The dual source housing fitted directly around the Zspray source of the Q-TOF with the reference sprayer positioned perpendicular to the analyte sprayer. During measurements, the reference spray was sampled every five seconds. Mass spectra were collected in the negative electrospray mode and acquired from 100 to 800 Da at an acquisition rate of 1 spectrum/s with an inter-acquisition delay of 0.1 s. The electrospray voltage was 2.3 kV, and cone voltages of 35 V and 60 V were applied to the analyte and reference materials, respectively, during acquisition. Accurate mass measurement was provided by infusing leucine enkephalin ($[M-H]^-$ = 554.2615) into the reference channel of the dual source. This 'lock mass' was infused at a flow rate of 3 µl/min using a KD Scientific (New Hope, PA, USA) Model 100 syringe pump at a concentration of 2 ng/µl leucine enkephalin in 50 % (v/v) methanol in water. Data acquisition and processing were carried out using Mass Lynx version 3.5. From the centroid data, which were obtained after the conversion of the continuum data acquired by the Q-oaTOF-MS, averaged and background-subtracted mass spectra of the compounds of interest were obtained. The accurate masses of the peaks of interest were entered into the Elecomp program, which is part of the Mass Lynx, to calculate possible elemental compositions. The parameter settings in the Elecomp program were: C 0-9 atoms, H 0-80 atoms, O 0-20 atoms, and S 0-1 atoms. The search window was 5 mDa, even-electron ions. The resulting elemental compositions were compared with the predicted conjugated compounds of polyphenols in the Ukogi extract. Liquid chromatographic separation was

performed on a Develosil ODS-SR (Nomura Chemical. Co., Ltd., Aichi, Japan, column size; 150 × 2.0 mm, particle size; 5 µm). An oven maintained the temperature of the column at 40 °C. Solvent A (water: CH_3COOH, 100:1, v/v) and B (acetonitrile: CH_3COOH, 100:1, v/v) were run at flow rate of 0.2 ml/min. The analysis was performed using a linear gradient from 20 to 45 % B for 30 min.

3. Results

3.1 Components of Ukogi powder

Different kinds of Ukogi powder were used for a single dose and a long-term multiple dose experiments. The content of rutin in Ukogi powder used in a single dose experiment was two times larger than that of Ukogi powder used in a long-term experiment. The polyphenols composition of the Ukogi extract used in the single does is shown in Figure 1. From the relationships between the retention time and negative ion mass $[M-H]^-$, the peaks were identified as chlorogenic acid (16.7 min), rutin (32.6 min), and kaempferol 3 *O*-rutinoside (36.7 min). These peaks also corresponded to the retention times of the reference samples and negative ion mass. The rats in the single does study consumed 0.5 g Ukogi powder: 12.8 mg chlorogenic acid, 5.3 mg rutin, 5.5 mg kaempferol 3-*O*-rutinoside. The rats in the long-term multiple does experiment consumed: chlorogenic acid, rutin, kaempferol 3-*O*-rutinoside, 107 mg, 21.8 mg, 44.1 mg over 8 days and 181 mg, 36.8 mg , 74.6 mg over 15 days.

3.2 Direct detection in non-hydrolyzed plasma

The hot water extract of Ukogi was administered orally to rats by gastric intubation. The plasma of rats was analyzed by LC/ESI-MS. Chlorogenic acid, rutin, and kaempferol 3-*O*-rutinoside in the plasma one hour after Ukogi extract administration were identified from the retention times and their ion peaks, which were comparable to those of the standard compounds.

Furthermore, quantitative analysis was performed for flavonoid glycoside with the more sensitive mode, that is, the SIM mode. Linear calibration curves were obtained in the range of 5-50 nM. According to the calculated area of each peak, the concentrations of rutin at 0.5 h and 1 h after consumption of Ukogi were estimated to be 13.6 ± 3.65 nM

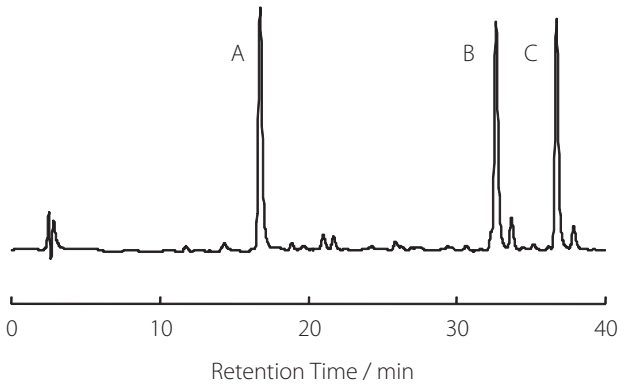

Figure 1: HPLC chromatogram of a hot water extract of Acanthopanax sieboldianus Makino. (A) chlorogenic acid, (B) rutin, and (C) kaempferol 3-*O*-rutinoside were confirmed from MS spectra and retention times compared with standards. The analysis was performed using a linear gradient from 5 to 25 % B for 40 min (solvent A: water/H_3PO_4 (100:0.1 v/v), solvent B: acetonitrile/H_3PO_4 (100:0.1 v/v)). Separation was achieved on an C30-UG-5 column (150 × 2.0 mm, 5 µm). The sample (2 µl) was injected.

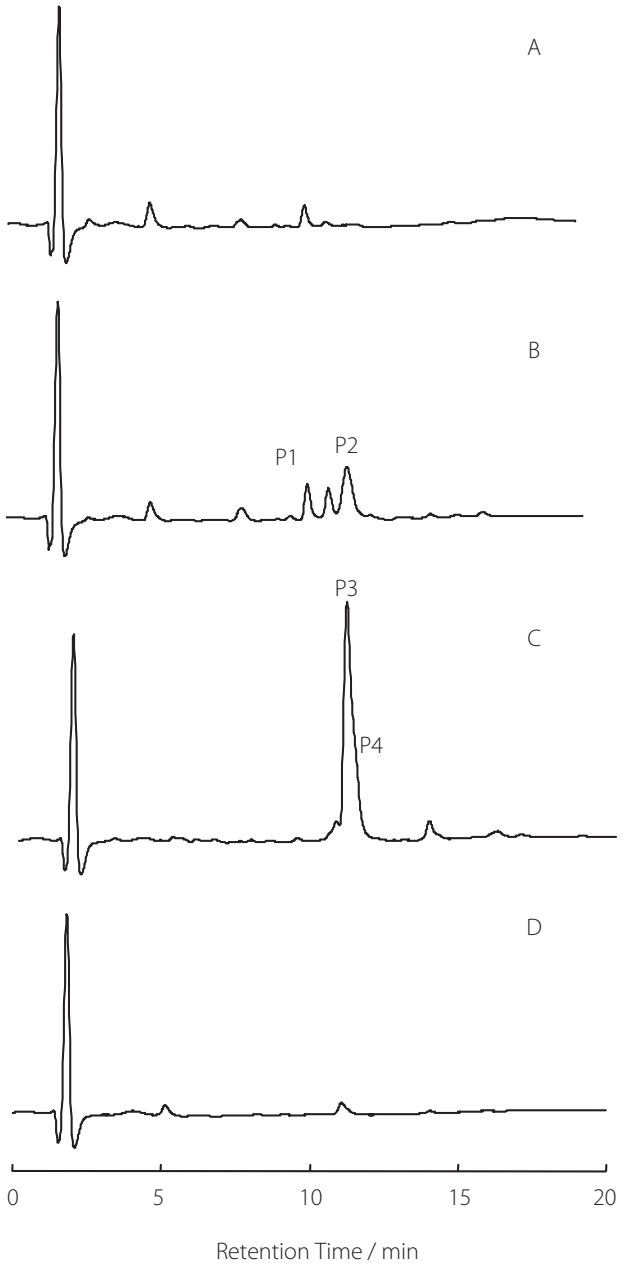

Figure 2: Plasma profiles after administration of Ukogi. (A) and (B) were 0.5 h,8 h after administration of Ukogi extract in single dose. (C) and (D) were 15 days of feeding 5 % Ukogi diet and control diet in long-term dose, respectively.

and 12.8 ± 2.43 nM, respectively. The quantity of kaempferol 3 *O*-rutinoside could not be estimated because peltatoside (I.S.) contained small amounts of it. The concentration of chlorogenic acid by LC-ECD at 0.5 h and 1 h after consumption of Ukogi were 0.528 ± 0.160 μM and 0.433 ± 0.102 μM, respectively. Chlorogenic acid and rutin were not detected in the plasma 2-24 h after dosing Ukogi.

Figure 2 shows the HPLC chromatogram of rat plasma; A: 0.5 hr after administration of Ukogi in single dose, B: 8 hr after administration of Ukogi in single dose, C: the plasma fed the 5 % Ukogi diet for 15 days and D: the plasma fed a normal diet in the long-term multiple doses. The HPLC profile B in single dose is characterized by the presence of two unidentified peaks, P1 (t_R 10.4) and P2 (t_R 11.8 min), corresponding to the conjugated metabolites of flavonoid. In long-term dose, two products were formed P3 and P4, which was a small shoulder on P3 in the HPLC chromatogram of C. P2 had maximum absorptions, 255, 351 nm. This spectrum was the same one of the large peak P3 (t_R 11.0). No free flavonoid agricons or chlorogenic acid were detected in non-hydrolyzed plasma from rats of long-term dose (limit detection was 0.05 μM). Rutin were also not detected by LC/ESI-MS-SIM. LC/ESI-MS analysis revealed that the peak P3 and P4 consisted of the sulfo-glucuronide of the corresponding kaempferol, which was molecular ion m/z 541 in the negative ion mode. The peak (m/z 541) was detected in the plasma of rats fed the 5 % Ukogi diet for 8 days and 24 days (data not shown). It was not detected in the Ukogi extract or control rat plasma. However, there was a lack of identification of unknown compounds in the plasma due to the lack of standard materials for comparison. Therefore, an exact mass and an exact mass MS/MS of this peak were examined in detail by LC/ESI/Q-oaTOF-MS.

Figure 3 shows the HPLC chromatogram, total ion chromatogram, and exact ion chromatograms of the plasma of rats fed the 5 % Ukogi diet for 24 days obtained by LC/ESI/Q-oaTOF-MS. The dominant ion peak was m/z 541 at 6.70 min and the small shoulders was m/z 571 at 6.90 min. The exact mass for metabolites at 6.72 minutes was obtained from the averaged and background-subtracted mass spectra in Figure 4. Each peaks of exact mass were m/z 541.0278 and m/z 571.0388, respectively. These ions were some degree of co-elution, then, they were not separated. This, however, posed no problem for exact mass measurement since they were well resolved by their individual masses. Next, the product ion spectra of m/z 541.0278 and m/z 571.0388 were obtained as shown in Figure 5. There were some ion peaks of kaempferol of m/z 285.0406, kaempferol sulfate of m/z 364.9975, and kaempferol glucuronide of m/z 461.0721 in the MS/MS of m/z 541.0278. On the other hand, there were some ion peaks of methylquercetin of m/z 315.0521, methylquercetin sulfate of m/z 395.0023, and methylquercetin glucuronide of m/z 491.0855 in the MS/MS of m/z 571.0388. The exact mass data obtained are summarized in Table 1. The results gave values that were in good agreement with the theoretical masses. The assignments of m/z 541.0278 and m/z 571.0388 were assumed to be kaempferol 3-*O*-sulfoglucuronide or kaempferol 3-*O*-glucurosulfate. Furthermore, the peak of m/z 571.0388 was identified as 3'-*O*-methylquercetin

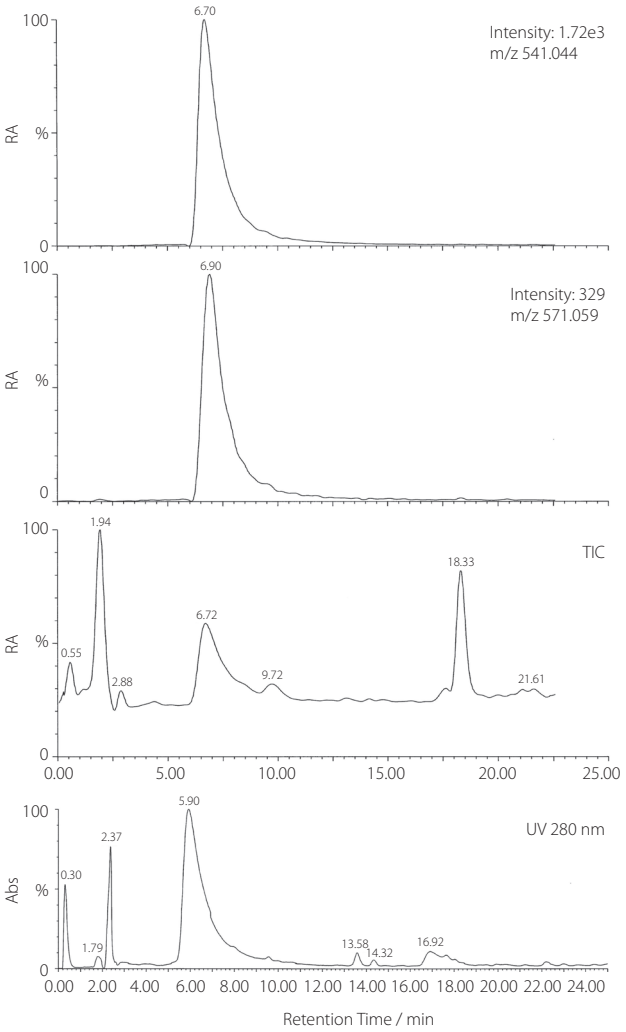

Figure 3: Exact ion chromatograms and HPLC chromatogram obtained by LC/ESI/Q-oaTOF-MS from the plasma after feeding the 5 % Ukogi diet for 24 days.

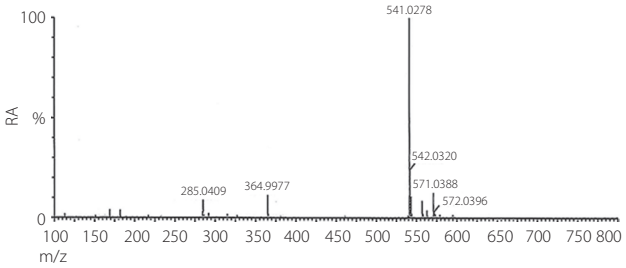

Figure 4: Exact mass spectra at 6.72 min for Figure 3 obtained by LC/ESI/Q-oaTOF-MS

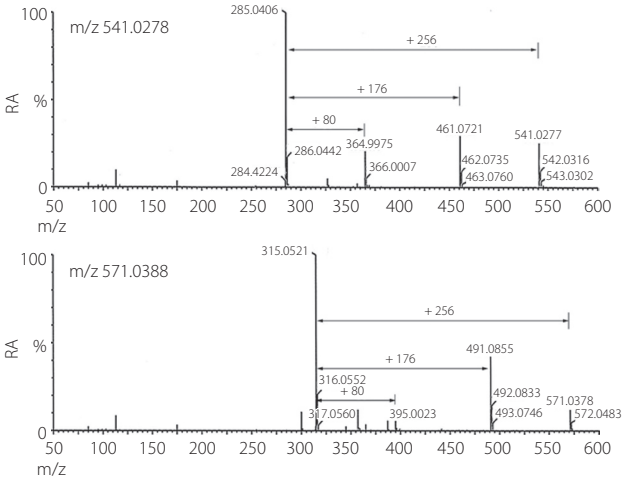

Figure 5: Product ion spectra of m/z 541.0277 and m/z 571.0388 obtained by LC/ESI/Q-oaTOF-MS.

sulfoglucuronide or 3'-*O*-methylquercetin glucurosulfate and methylquercetin sulfoglucuronide or methylquercetin glucurosulfate.

4. Discussion

In vitro antioxidant potential of fruit and vegetables plainly reflects their phenolic composition and vitamin C content (Proteggente et al., 2002). However, the functions of flavonoids *in vivo* were not discussed in term of conjugated or metabolized forms of dietary phenolics. Then, it is important to clarify the function of polyphenols from bioavailability of polyphenol.

Detection of intact chlorogenic acid and rutin in plasma 0.5 h after dosing Ukogi shows that it has been absorbed in its native form. The low recovery of these compounds were thought to be metabolized other compounds. Recently, Gonthier et al. (2003) reported that chlorogenic acid was degradated by gut microflora and the metabolites were mainly *m*-coumaric acid and derivaties of phenylpropionic, benzoic and hippuric acids in both urine and plasma. Therefore, the difference of chemical structure depended on their rate and the extent of intestinal absorption and the nature of the metabolites circulating in the plasma.

We have paid attention to the fact that the antioxidant activities of polyphenol compounds *in vitro* in the aqueous phase were evaluated by the superoxide scavenging abilities and inhibitory effects on XOD (Kaneyuki et al., 1999; Cos et al., 1998; Cotelle et al., 1992; Okuda et al., 1994; Aucamp et al., 1997; Koide et al., 2000) which generates superoxide from oxygen and the nucleic acid. The superoxide scavenging abilities of molecules with the same molecular frame depended on the number of their hydroxyl groups. Pyrogallol, catechol, and monophenol structures showed the highest ability (in decreasing order). The loss of a phenolic OH group of the B ring of quercetin by methylation led to a dramatic decrease in superoxide scavenging ability. On the other hand, the inhibitory effect on XOD also depended on the chemical structure. The superoxide scavenging ability of Ukogi *in vitro* is high level in other vegetables in our data. It is the most strong of those of popular vegetables, such as spinach or garland chrysanthemum. It mainly depended on the amount of chlorogenic acid (260 mg/ 100 g FW) which has dihydroxyl structure. Ukogi is also flavonol-rich vegetables (100 mg/100 g FW) at the same level as onion or any more. In term of the metabolism in plasma after administration of Ukogi extract, at an early stage of postabsorption, the metabolites of flavonols and chlorogenic acid seem to be useful as antioxidant in term of having superoxide scavenging ability, whereas, at later stage, only the flavonols seem to be.

We couldn't identify directly the flavonols metabolite after administration of Ukogi in single dose. Moon et al. (2001) identified the metabolite in the rat plasma 30 min after oral administration of quercetin (250 mg /kg body weight) as quercetin 3-O-β-D-glucuronide (Q3GA) and quercetin 4'-O-β-D-glucuronide (Q4'GA). Then, the metabolites 0.5 h after administration of Ukogi in single dose might be suspected to kaempferol-3-glucuronide or those quercetin metabolites. Day also reported that quercetin, quercetin -4'-glucuronide and quercetin-3'-glucuronide were potent inhibitors of

Table 1: Exact mass results for metabolites of rat plasma administrated the 5 % Ukogi diet for 24 days.

Components	Mass measured	Theoretical mass	Deviation (mDa)	Expected formula
kaempferol sulfo-glucuronide	541.0277	$[M-H]^-$ 541.0288	-1.1	$C_{21}H_{17}O_{15}S$
kaempferol	285.0406	$[M-H]^-$ 285.0399	0.7	$C_{15}H_9O_6$
kaempferol sulfate	364.9975	$[M-H]^-$ 364.9967	0.8	$C_{15}H_9O_9S$
kaempferol glucuronide	461.0721	$[M-H]^-$ 461.0720	0.1	$C_{21}H_{17}O_{12}$
methylquercetin sulfo-glucuronide	571.0388	$[M-H]^-$ 571.0394	-0.6	$C_{22}H_{19}O_{16}S$
methylquercetin	315.0521	$[M-H]^-$ 315.0505	1.6	$C_{16}H_{11}O_7$
methylquercetin sulfate	395.0023	$[M-H]^-$ 395.0073	-5.0	$C_{16}H_{11}O_{10}S$
methylquercetin glucuronide	491.0855	$[M-H]^-$ 491.0826	2.9	$C_{22}H_{19}O_{13}$

on xanthine oxidase (Day et al., 2000). Then, some of the metabolite in single dose could still suppress superoxide generation in the blood.

The concentrations of flavonols metabolites increased much than that of metabolites of chlorogenic acid by repeating ingestion of the Ukogi powder over time. The conjugated metabolites in the plasma of rats administered a 5 % Ukogi diet for 24 days were identified kaempferol sulfo-glucuronide and 3'-*O*-methylquercetin sulfo-glucuronide by LC/ESI/Q-oaTOF-MS. They are thought to be the final products of the absorbed rutin and kaempferol 3-*O*-rutinoside contained in Ukogi. The metabolites might have neither superoxide scavenging ability nor inhibitor of xanthine oxidase according to the position of conjugation. However, it was reported by Shimoi et al., (2001) that quercetin glucuronides could be deconjugated at the site of action (e.g. the endothelium). If it is possible, the metabolite would be useful as inhibitor of XOD in the body.

The glucuronidation, sulfation, and methylation of quercetin will modify the antioxidant activity due to the hydrophobicity and electron delocalization of the metabolites. The metabolite may serve to decrease the superoxide concentration in the body, when the metabolite binds to XOD in the blood. The metabolite was associated with an increase in plasma antioxidant potential and may therefore have an important role to play in the modulation of exposure to cellular oxidative stress. Overall, intake of Ukogi may have a beneficial effect in the body via antioxidation in that accumulated flavonoid metabolites not only scavenge superoxide but also block superoxide generation. In the future, the role of these metabolites as alternative candidates for antioxidants should be clarified in the vascular system.

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