Identification and Antioxidant Activity of Flavonoid Metabolites in Plasma and Urine of Eriocitrin-Treated Rats

Yoshiaki Miyake,*,† Kayoko Shimoi,‡ Shigenori Kumazawa,‡ Kanefumi Yamamoto,† Naohide Kinae,‡ and Toshihiko Osawa§

Fundamental Technical Laboratory of Pokka Corporation, Ltd., 45-2 Kumanosyo, Shikatsu-cho, Nishikasugai-gun, Aichi, 481-8515, Japan, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan, and Laboratory of Food Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Chikusa, Nagoya 464-8601, Japan

Eriocitrin, a flavonoid glycoside present in lemon fruit, is metabolized in vivo to a series of eriodictyol, methylated eriodictyol, 3,4-dihydroxyhydrocinnamic acid, and their conjugates. Plasma antioxidant activity increased following oral administration of aqueous eriocitrin solutions to rats. Eriocitrin metabolites were found in plasma and renal excreted urine through HPLC and LC−MS analyses. Eriocitrin was not detected in plasma and urine, but eriodictyol, homoeriodictyol, and hesperetin in their conjugated forms were detected in plasma of 4.0 h following administration of eriocitrin. In urine for 24 h, both nonconjugates and conjugates of these metabolites were detected. 3,4-Dihydroxyhydrocinnamic acid, which is metabolized from eriodictyol by intestinal bacteria, was detected in slight amounts with each form in 4.0-h plasma and 24-h urine. Eriocitrin was suggested to be metabolized by intestinal bacteria, and then eriodictyol and 3,4-dihydroxyhydrocinnamic of its metabolite were absorbed. Following administration of eriocitrin, plasma exhibited an elevated resistance effect to lipid peroxidation. Eriocitrin metabolites functioning as antioxidant agents are discussed.

Keywords: *Flavonoids; eriocitrin; antioxidant; metabolism; rats*

INTRODUCTION

Flavonoids are widely distributed in plant foods such as vegetables and fruits. They possess a unique C6−C3−C6 structure (diphenylpropane structure) with phenolic hydroxy groups; more than 4000 different functional group substitution patterns have been identified as natural flavonoids. The average intake of flavonoids in Western diets was estimated to be 1 g/day (Kuhnan, 1976). Flavonoids in citrus fruits are known as bioflavonoids or vitamin P, which exhibit beneficial effects on capillary permeability and fragility (Rusznyak and Szent-Gyorgyi, 1936). These compounds have been investigated regarding their physiological functions such as antiinflammatory, anticarcinogenic, and antitumor activities (Bracke et al., 1994; Middleton and Kandaswami, 1994; Attaway, 1994).

Much attention has been focused on the involvement of oxidation stress by active oxygen species and free radicals in aging and disease. The active oxygen species have been proposed as the attacking agents on polyunsaturated fatty acid (PUFA) in cell membranes. Lipid peroxidation is suspected to be strongly associated with aging and carcinogenesis (Cutler, 1992). The antioxidants of food are thought to prevent diseases caused by oxidative stress (Cutler, 1984; Frankel et al., 1993). The antioxidant activity of flavonoids has attracted much attention in relation to their physiological functions. Dietary flavonoids are considered to aid in the prevention of coronary heart disease because epidemiological studies have shown an inverse relationship between the intake of dietary flavonoids and coronary heart disease (Hertog et al., 1993). The so-called French paradox is at least partially related to the consumption of red wine rich in flavonoids and other phenolic compounds. It is, however, necessary to know the biodynamics of flavonoids after intake for estimation of in vivo antioxidant activity.

Flavonoids in lemon fruit were first reported to exhibit beneficial effects on capillary permeability and fragility together with ascorbic acid (Rusznyak and Szent-Gyorgyi, 1936). We have paid attention to lemon flavonoids and are involved in the isolation of antioxidative flavonoid glycosides from lemon fruit and have identified eriocitrin (eriodictyol 7-*O*-*β*-rutinoside) of the flavanone glycoside (Miyake et al., 1997a) and 6,8-di-*C*-*β*-glucosyldiosmin and 6-*C*-*β*-glucosyldiosmin of the *C*-glucosylflavones (Miyake et al., 1997c). Eriocitrin had a stronger antioxidant than the other citrus flavonoid compounds and was abundantly present in lemon and lime fruits (Miyake et al., 1997a, 1998). It is important to determine how the antioxidants in food are metabolized in vivo and how antioxidant metabolites function in a living system. The antioxidative mechanism of curcumin in turmeric in vivo was reported to be absorbed from the digestive tract after it was metabolized to a higher antioxidative tetrahydrocurucumin by intestinal bacteria (Sugiyama et al., 1996). We reported

* To whom correspondence should be addressed. Tel: 011-81-568-21-1126. Fax: 011-81-568-21-4331. E-mail: yoshiaki_miyake@mr.pokka.co.jp.

† Fundamental Technical Laboratory of Pokka Corporation, Ltd.

‡ University of Shizuoka.

§ Nagoya University Graduate School of Bioagricultural Sciences.

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the metabolic mechanism of eriocitrin by the in vitro experiment using human intestinal bacteria (Miyake et al., 1997b). It is very important to evaluate the bioavailability of flavonoids in order to clarify whether the absorbed flavonoids function as antioxidants in vivo. Quercetin, which is a flavonoid in onion, was reported to be absorbed, and its bioavailability in humans was examined (Hollman et al., 1997). It has been reported that luteolin was absorbed and converted to glucuronides passing through the intestinal mucosa and that the main circulating conjugate was a monoglucuronide, although some luteolin avoids the fate of glucuronidation, sulfation, and/or methylation (Shimoi et al., 1998).

In this study, we investigated the metabolism of eriocitrin in rats in vivo. Following oral administration of eriocitrin, metabolites in the plasma and the renal excreted urine were examined by HPLC and LC−MS (liquid chromatography−mass spectrometry) analysis.

MATERIALS AND METHODS

Materials and Chemicals. Eriocitrin was prepared from lemon peel extract (Miyake et al., 1997a), and the purity was greater than 99.0% as determined by HPLC. Other flavonoids were purchased from Funakoshi Ltd., Tokyo, Japan. 3,4-Hihydroxyhydrocinnamic acid (3,4-DHCA) was purchased from Aldrich Chemical Co., Milwaukee, WI. β-Glucuronidase and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Sulfatase type H-5 was obtained from Sigma Chemical Co., St. Louis, MO. Other reagents were of commercial grade, and all solvents used were of HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Animals and Diets. Male Sprague−Dawley rats (5 weeks old, Japan SLC, Ltd., Hamamatus, Japan), weighing 195−210 g, were housed in an air-conditioned room and fed CE-2 commercial food pellets (Crea Japan, Ltd., Tokyo, Japan) ad lib. One week before an experiment, the diet was changed to a synthetic basic diet consisting of 38% corn starch, 25% casein, 10% α-starch, 8% cellulose powder, 6% minerals, 5% sugar, 2% vitamins, and 6% lard (Oriental Yeast, Ltd., Tokyo, Japan). Rats were maintained and handled according to prescribed procedures (Guidelines for the Regulation of Animal Experimentation Committee, University of Shizuoka).

Sample Preparation of Blood and Urine. Rats (n = 5/group), which fasted overnight, were anesthetized with ether at different times of 0.5, 1.0, 2.0, and 4.0 h following administration of eriocitrin (75 μmol/kg in distilled water) through gastric intubation, and blood was withdrawn from the abdominal aorta into heparinized tubes. Rat plasma from nontreated specimens was used as a control or 0-h plasma. On the other hand, the urine for 24 and 48 h following administration of eriocitrin (50 μmol/kg in distilled water) was collected (n = 4/group). Plasma and urine samples were analyzed by HPLC.

Absorption Experiment with Everted Intestine. The intestinal absorption of eriocitrin from the mucosa to the serosal side was studied according to the previously described method (Shimoi et al., 1998). Briefly, rats that fasted overnight were anesthetized with pentobarbital, and six consecutive segments of 2.5 cm each were dissected from the middle part of the upper half (jejunum) of the small intestine and rinsed with a Ringer's solution containing 140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM KH_2PO_4, and 1 mM glucose (pH 7.4). They were everted and fixed over a fenestrated conical polypropylene tube attached with Tygon tubing. These sacs were placed in 5 mL of Ringer's solution containing eriocitrin, and the serosal side was filled with 0.3 mL of Ringer's solution. They were incubated bubbling with 95% O_2 and 5% CO_2 at 37 °C. The medium of the serosal side was substituted with fresh Ringer's solution 15 min after the start of incubation.

HPLC Analysis of Eriocitrin Metabolites in Plasma and Urine. The sample (0.5 mL) of plasma, urine, and the incubation medium of the serosal side were acidified with the same volume of 0.01 M oxalic acid. This solution was applied to a Sep-Pak C_{18} cartridge. After the cartridge was washed with 0.01 M oxalic acid and distilled water, the methanol eluate was obtained. The eluate was evaporated to dryness, and the residue was dissolved in 100 μL of methanol. After centrifugation for 2 min at 4 °C and 20000g, the supernatants were used for HPLC analysis. The flavonoids of eriocitrin metabolites were analyzed chromatographically with a Shimadzu system (Kyoto, Japan) using a YMC-Pack ODS column (YMC Co., Ltd., Kyoto, Japan, column size; ϕ 4.6 × 250 mm, particle size; 5 μm) and UV detection (333 nm). The mobile phase contained the following: solvent A, methanol; solvent B, distilled water with 5% acetic acid. Initial concentration of solvent A:solvent B (20:80) was changed in a gradient toward that of solvent A:solvent B (70:30) for 30 min. The column temperature was maintained at 40 °C, and the flow rate was 1.0 mL/min. 3,4-DHCA was determined with the same HPLC system using an electrochemical detector (750 mV), and the mobile phase contained the following: solvent C, methanol; solvent D, 20 mM sodium trichloroacetate buffer (pH 2.5) with 0.1 mM EDTA·2Na. Initial concentration of solvent C:solvent D (30:70) was changed in a gradient toward that of solvent C:solvent D (80:20) for 40 min.

For detection of the conjugates, each sample was acidified with 1 M acetate buffer (pH 4.5) and was preincubated for 2 min at 37 °C. Solutions were treated with 5.4×10^2 units/mL of β-glucuronidase and 0.2×10^2 units/mL of sulfatase for 20 min at 37 °C, and then the same volume of 0.01 M oxalic acid was added. The mixtures were centrifuged for 5 min at 10000g. Supernatants were prepared and analyzed in the same way as described above. The contents of the sample obtained from treatment with β-glucuronidase/sulfatase were expressed as the sum amount of the nonconjugates of free compounds and the conjugates.

LC−MS Analysis. LC−MS (electrospray ionization method, negative mode) analysis was performed in a Thermo Quest LCQ mass spectrometer with a Shiseido liquid chromatograph S1-1 apparatus. The LC conditions were as follows: column, a YMC-Pack ODS column (YMC Co., Ltd., Kyoto, Japan, column size; ϕ 4.6 × 250 mm, particle size; 5 μm); detection, UV 333 nm; flow rate, 0.5 mL/min; the same as in the HPLC flavonoid analysis except for the gradient conditions. The analysis was carried out at 40 °C, and the mobile phase used the above-described solvents A and B. Initial concentration of solvent A:solvent B (30:70) was changed in a gradient toward that of solvent A:solvent B (80:20) for 30 min.

Measurement of Oxidation Resistance of the Plasma. The plasma was oxidized at 37 °C by incubation with AAPH (Frei et al., 1988). The final concentration of AAPH was 25 mM. After an incubation period of 7 h, lipid peroxidation was stopped by the addition of butylated hydroxytoluene in a final concentration of 60 μM. The degree of oxidation was immediately measured by the thiobarbituric acid reactive substances (TBARS) assay (Yagi, 1976).

Determination of Antioxidants in Plasma. The antioxidants of α-tocopherol, ascorbic acid, uric acid, and bilirubin in plasma were determined. The sample of plasma (50 μL) was mixed with methanol (200 μL), and the mixture was centrifuged at 20000g for 3 min. The supernatant was added to hexane (500 μL) and centrifuged at 20000g for 3 min after being mixed. The 5 μL of supernatant was analyzed with HPLC for determination of α-tocopherol (Silva et al., 1998). HPLC was used as in the above conditions except for a solvent phase of 55% acetonitrile and 45% ethanol with a fluorescence detector (ex, 295 nm; em, 325 nm). As for the contents of ascorbic acid, the plasma (100 μL) was added to methanol (400 μL), mixed, and centrifuged at 20000g for 3 min. The supernatant (20 μL) was analyzed with HPLC using a polyamine column (ϕ 4.6 × 250 mm, 5 μm, YMC), 34 °C, 1 mL/min, a solvent of 75% acetonitrile and 25% $NH_4H_2PO_4$, and a detector of 450 nm (Yamamoto and Ames, 1987). The uric acid and bilirubin in the plasma were enzymatically measured using commercial kits (Uric acid B-Test Wako, Bilirubin B II-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan)

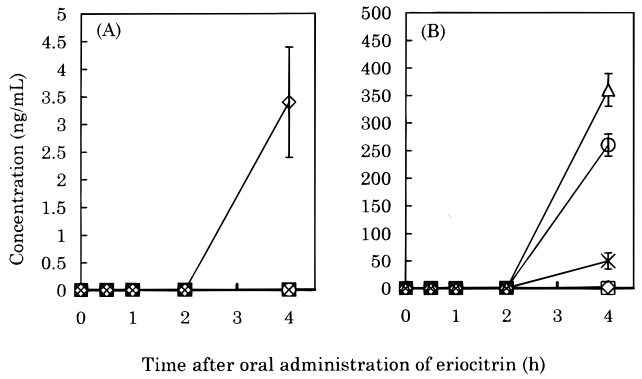

Figure 1. Contents of eriocitrin metabolites in rat plasma following oral administration of eriocitrin. The plasma concentration of metabolites in the free form (A) and the conjugated form (B) at different times following administration of eriocitrin (75 μmol/kg in distilled water) were determined by HPLC analysis as described in Materials and Methods (n = 5): eriocitrin, □; eriodictyol, △; homoeriodictyol, ○; hesperetin, ×; 3,4-DHCA, ◇.

by the colorimetric method (Henry et al., 1957; Malloy and Evelyn, 1937).

Statistical Analyses. The statistical significance of the results was analyzed through an analysis of variance (ANOVA) followed by Turkey's multiple comparison test. Differences were considered significant at $p < 0.05$. Each value represents a mean of ± SD. (n = 4−5).

RESULTS

Identification of Eriocitrin Metabolites in Rat Plasma. Eriocitrin and its metabolites in rat plasma of different times following oral administration of eriocitrin were determined by HPLC analysis (Figure 1). Their compounds were examined to determine whether the free form was a nonconjugate (Figure 1A) or the glucuro and/or sulfo conjugates (Figure 1B). The content of the latter was determined by the treatment of β-glucuronidase/sulfatase. Eriocitrin was not detected in plasma following administration of eriocitrin, but eriodictyol, homoeriodictyol, and hesperetin were detected in the 4.0-h plasma treated with β-glucuronidase/sulfatase. They exist in the form of glucuro and sulfo conjugates because they were not detected without treatment of β-glucuronidase/sulfatase. Eriodictyol is the aglycone of eriocitrin. Homoeriodictyol is methoxylated at the 3′ position of the B ring of eriodictyol, and hesperetin is methoxylated at the 4′ position of the B ring of eriodictyol. These metabolites in plasma of 4.0 h following administration of eriocitrin were also identified through LC−MS analysis (data not shown). 3,4-DHCA, which was metabolized from eriodictyol by intestinal bacteria (Hollman et al., 1997), was detected in slight amounts with the free and conjugated forms in plasma of 4.0 h following administration of eriocitrin. Furthermore, the two unknown peaks, which are different from eriocitrin, eriodictyol, homoeriodictyol, hesperetin, and 3,4-DHCA, were detected in plasma for 0.5−2.0 h following administration of eriocitrin through treatment with glucuronidase/sulfatase by HPLC analysis and were shown to be maximized in the contents of 0.5-h plasma. The retention time of these peaks for the HPLC profile was detected at nearly that of eriocitrin. These peaks could not be measured for molecular weight using LC−MS analysis because of their low abundance.

Identification of Eriocitrin Metabolites in Rat Urine. Eriocitrin metabolites in renal excreted urine following oral administration were examined using HPLC analysis (Figure 2). Both nonconjugates and conjugates of eriodictyol, homoeriodictyol, and hesperetin were detected in urine for 24 h, and their metabolites in urine for 48 h were not detected. 3,4-DHCA was also detected in slight amounts in each form in urine for 24 h. Eriodictyol, homoeriodictyol, and hesperetin of eriocitrin metabolites in the urine for 24 h were identified through LC−MS. The profile of the sample, which was treated with β-glucuronidase/sulfatase, is shown in Figure 3. It was confirmed that the peak with the scan mode of $(M - H)^-$ ion 287 was compatible with that of eriodictyol with UV detection (Figure 3C) and that the peak with the scan mode of $(M - H)^-$ ion 301 was compatible with that of homoeriodictyol and hesperetin with UV detection, respectively (Figure 3D). Peaks a−c in Figure 3C,D were also identified for eriodictyol, homoeriodictyol, and hesperetin from their correspondence with a mass spectra of standard samples (Figure 4).

The absorption ratio of eriocitrin in vivo was calculated from the sum content of these eriocitrin metabolites, which are detected in both free and conjugated forms of eriodictyol, homoeriodictyol, hesperetine, and 3,4-DHCA in renal excreted urine, against the amount of oral eriocitrin administered to the rats. The total flavonoid absorption for the treated rats was 5.68% ± 0.32.

Absorption of Eriocitrin with Everted Intestine. The absorption of eriocitrin through the small intestine was examined through experiments using an everted intestine. The constituents appearing in the serosal side after intestinal absorption of eriocitrin did not detect eriocitrin and its metabolites such as eriodictyol, homoeriodictyol, hesperetin, and 3,4-DHCA with either no-treatment or treatment with glucuronidase/sulfatase using HPLC analysis. But the two unknown peaks, which were detected in the 0.5−2.0-h plasma following administration of eriocitrin with a treatment of glucuronidase/sulfatase, were detected in the serosal side with a treatment of glucuronidase/sulfatase using HPLC analysis.

Susceptibility of Rat Plasma to Lipid Peroxidation by Administration of Eriocitrin. We examined the susceptibility to lipid peroxidation of rat plasma following administration of eriocitrin to determine if eriocitrin metabolites in plasma express antioxidative activity. The plasma of 0.5, 1.0, 2.0, and 4.0 h following administration of eriocitrin showed significantly less oxidation as compared with that of the 0-h plasma used as a control throughout the 7-h incubation when the lipid peroxidation was induced by AAPH (Table 1). Furthermore, we examined the contents of antioxidants such as α-tocopherol, ascorbic acid, uric acid, and bilirubin in the plasma following administration of eriocitrin because of the possibility that these antioxidants participated in creating an effect of resistance to lipid oxidation by an APPH radical. However, the significant differences of the contents of these antioxidants in all examined plasma were not observed (Table 2).

DISCUSSION

As shown in Figure 5, we proposed the metabolic process of eriocitrin in vivo from the result, where eriocitrin metabolites were identified in plasma and renal excreted urine of orally administrated rats using

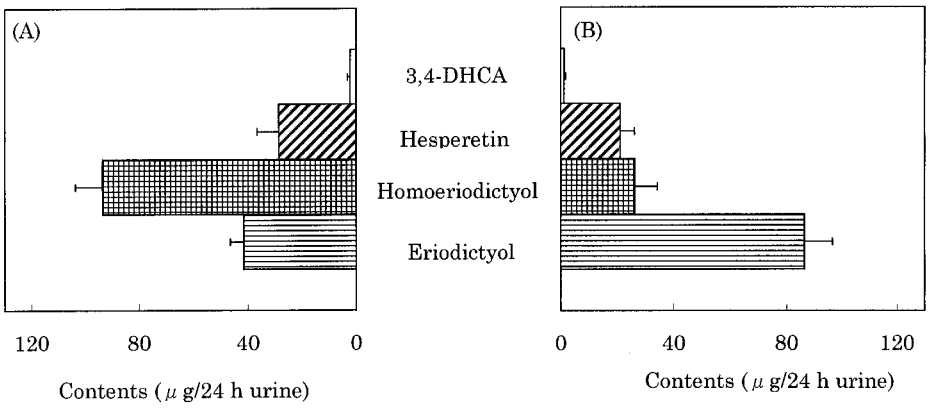

Figure 2. Contents of eriocitrin metabolites in rat urine excreted for 24 h following oral administration of eriocitrin. Eriocitrin metabolites of the free form (A) and the conjugated form (B). Eriocitrin (50 μmol/kg in distilled water) was administered by gastric intubation. For determination of the metabolites in the conjugated form, urine samples were treated with β-glucuronidase/sulfatase ($n = 4$).

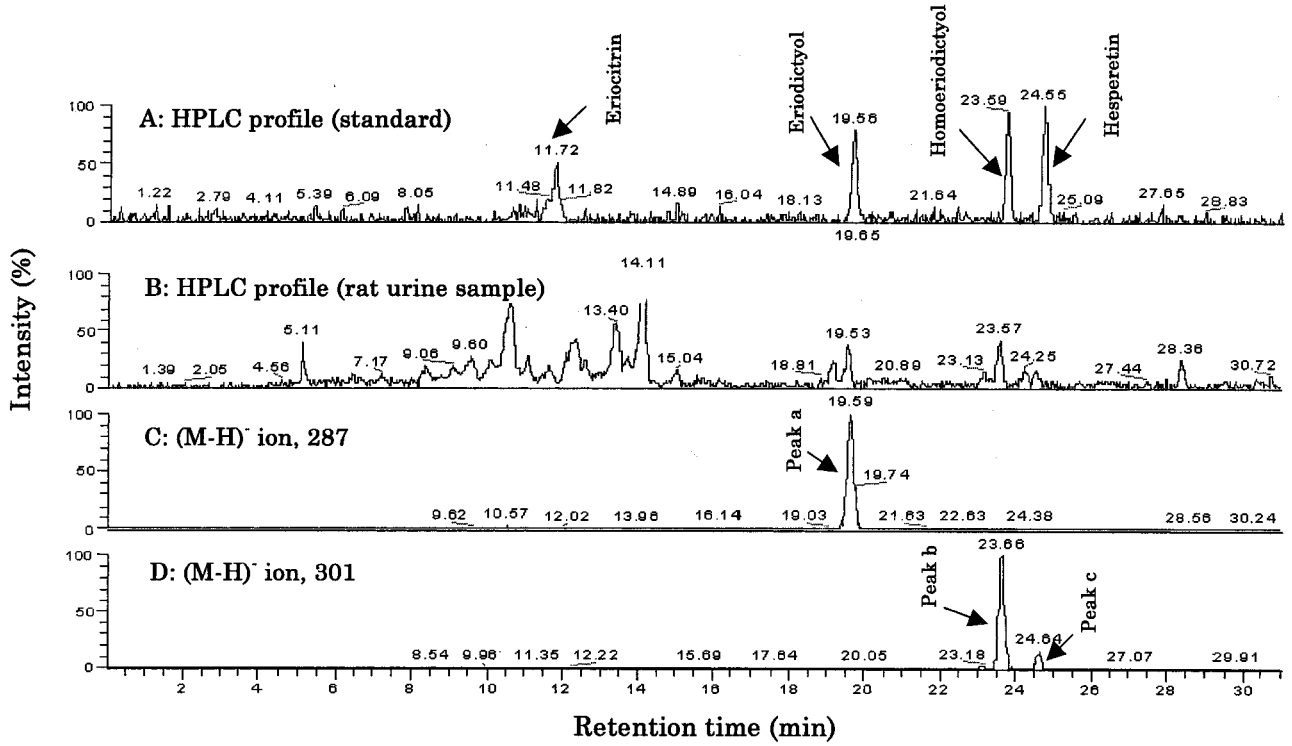

Figure 3. LC−MS analyses of eriocitrin metabolites in renal excreted urine for 24 h. The renal excreted urine for 24 h following administration of eriocitrin was treated with β-glucuronidase/sulfatase and the sample was determined through LC−MS analysis as described in Materials and Methods. HPLC profile of a standard sample (eriocitrin, eriodictyol, homoeriodictyol, and hesperetin) and a rat urine sample by HPLC analysis using detection of UV absorbance (A and B). Ion chromatogram of rat urine sample scanned by $(M - H)^-$ ion 287 for detection of eriodictyol (C) and by $(M - H)^-$ ion 301 for detection of homoeriodictyol and hesperetin (D).

HPLC or LC−MS analysis. The β-glycoside bond for a sugar-flavonoid bond of flavonoid glycosides is thought to be resistant to hydrolysis by pancreatic enzymes, and it has been shown that flavonoid glycosides are first hydrolyzed by digestive microflora before being absorbed (Kuhnan, 1976). We had reported that eriocitrin was metabolized to eriodictyol of its aglycone by *Bacteroides distasonis* or *Bacteroides uniformis* of intestinal bacteria and that eriodictyol was converted to 3,4-DHCA by *Clostridium butyricum* (Hollman et al., 1997). Additionally, we analyzed digested products of rats following administration of eriocitrin by HPLC and identified eriodictyol and 3,4-DHCA in the large intestines. In the absorption experiment with an everted intestine, eriodictyol and 3,4-DHCA of eriocitrin metabolites were not detected in the serosal side. However, eriodictyol and 3,4-DHCA were detected in the plasma (Figure 2). These results suggest that eriodictyol and 3,4-DHCA of eriocitrin metabolites were absorbed from the intestine after eriocitrin was metabolized to these metabolites by intestinal bacteria. The administration of rutin (quercetin 3-*O*-β-rutinoside) in rats has been reported to be detected as quercetin, its aglycone, in plasma of the treatment of β-glucuronidase/sulfatase and detected more slowly than that of quercetin because it must be hydrolyzed by cecal microflora (Manach et al., 1997). Eriodictyol, homoeriodictyol, and hesperetin of the conjugated form were detected in plasma of 4.0 h following administration of eriocitrin. Eriodictyol seems to be absorbed slowly because it is absorbed in the

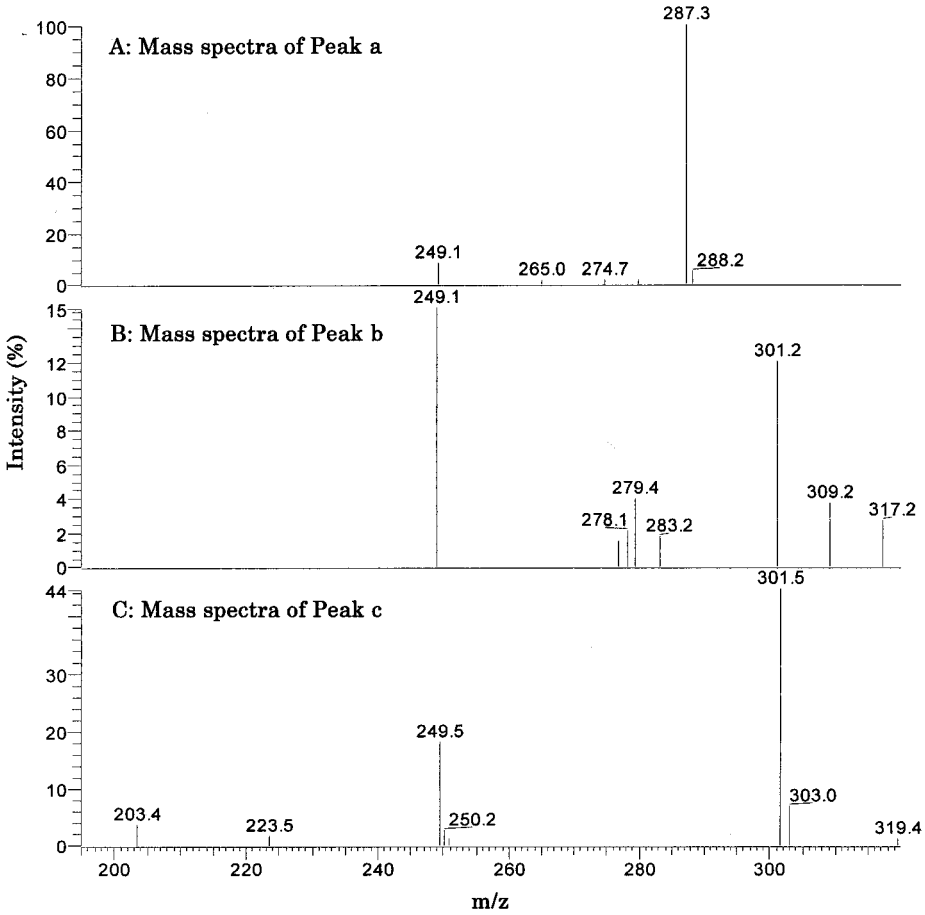

Figure 4. Mass spectra of eriocitrin metabolites in renal excreted urine for 24 h using LC−MS analyses. Mass spectra of peaks a−c in Figure 3C,D were shown with those of panels A−C, respectively.

Table 1. Susceptibility of Plasma of 0, 0.5, 1.0, 2.0, and 4.0 h to Lipid Peroxidation by Administration of Eriocitrin[a]

	TBARS (nM MDA/mL of blood)				
	time after oral administration of eriocitrin (h)				
time after addition of AAPH	0	0.5	1.0	2.0	4.0
0	1.3 ± 0.07[a]	1.4 ± 0.16[a]	1.1 ± 0.08[a]	1.0 ± 0.27[a]	1.1 ± 0.10[a]
7	7.6 ± 0.73[a]	5.7 ± 0.29[b]	5.4 ± 0.72[b]	5.4 ± 0.32[b]	5.6 ± 0.44[b]

[a] Values are means ± SD ($n = 5$). Values within the same row that do not share a common superscript letter are significantly different at $p < 0.05$.

Table 2. Content of Antioxidants in Plasma of 0, 0.5, 1.0, 2.0, and 4.0 h Following Oral Administration of Eriocitrin[a]

	time after oral administration of eriocitrin (h)				
	0, μg/mL	0.5, μg/mL	1.0, μg/mL	2.0, μg/mL	4.0, μg/mL
α-tocopherol	1.7 ± 0.1	1.7 ± 0.3	1.8 ± 0.1	1.6 ± 0.1	1.7 ± 0.1
ascorbic acid	2.4 ± 0.1	2.3 ± 0.2	2.1 ± 0.1	2.1 ± 0.3	2.0 ± 0.5
uric acid	15.0 ± 1.0	17.0 ± 2.0	13.5 ± 1.5	14.5 ± 3.5	14.0 ± 2.0
billirubin	1.2 ± 0.3	1.3 ± 0.4	1.4 ± 0.5	0.9 ± 0.4	0.8 ± 0.2

[a] Values are means ± SD ($n = 5$).

intestine after metabolism of intestinal bacteria and to be metabolized to homoeriodictyol and hesperetin by methoxylation in the liver. We estimated that the intestinal absorption mechanism of eriodictyol and 3,4-DHCA of eriocitrin metabolites resemble that of rutin metabolites. However, the two unknown peaks were detected in the serosal side with the absorption experiment of the everted intestine using HPLC analysis and maximally in the plasma of 0.5 h following administration of eriocitrin. The compounds of these peaks seem to be metabolized from eriocitrin and to be absorbed quickly in the small intestine. The amount in rat plasma following administration of eriocitrin seems to be significantly smaller than that of eriodictyol, homoeriodictyol, and hesperetin because we could not measure the molecular weight through LC−MS analysis in an amount less than that detected. The identification of their structures is required to solve the metabolic process of eriocitrin.

Furthermore, quercetin glucosides appear to be absorbed more easily than the aglycone from an experiment of healthy ileostomy volunteers with an intake of quercetin glucoside-rich onion (Hollman et al., 1995). αG-Rutin, which is formed from rutin by enzymatic transglycosylation, was also reported to be absorbed quickly in animals and to be detected in plasma in a

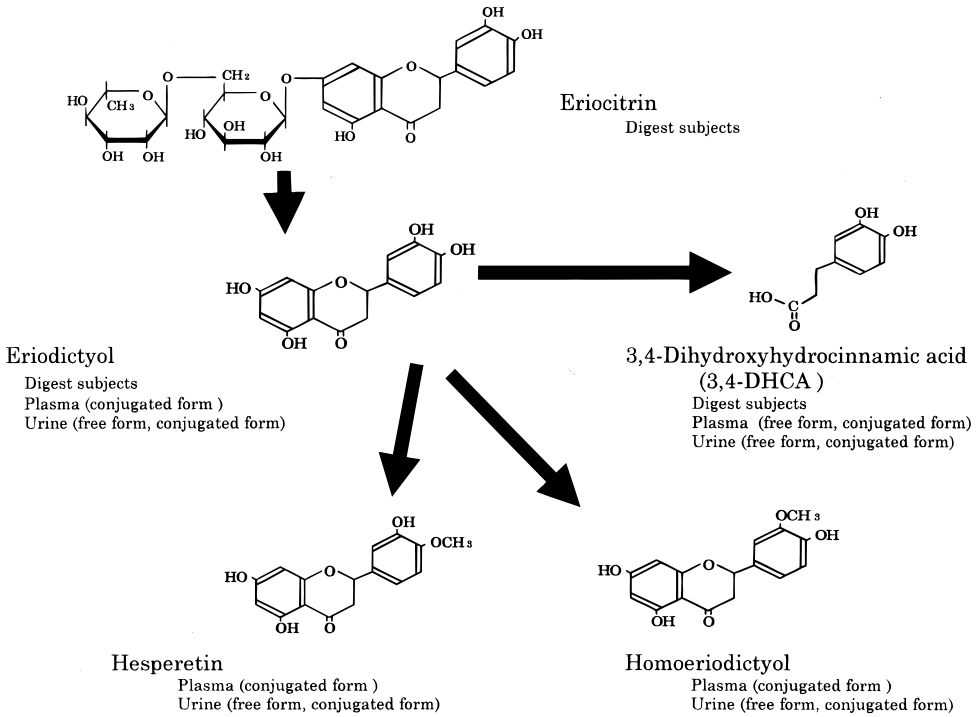

Figure 5. Scheme for the eriocitrin metabolic pathway in vivo in a rat. Eriocitrin (eriodictyol 7-*O*-*β*-rutinoside) is a flavonoid glycoside. The connected position of glucuronic acid and/or sulfate on conjugated metabolites (eriodictyol, homoeriodictyol, hesperetin, and 3,4-dihydroxyhydrocinnamic acid) was unknown.

short span of less than 30 min following oral administration (Shimoi et al., 1997). The oral administration of cyanidin 3-*O*-*β*-D-glucoside to rats showed that a small amount was detected and that protocatecuic acid, which is metabolized from the cyanidin of its aglycone, was detected as one of the main metabolites (Tsuda and Osawa, 1998). It has been reported that luteolin 7-*O*-*β*-glucoside was hydrolyzed to aglycone and then it was absorbed from the small intestine (Shimoi et al., 1998). Moreover, it has been reported that some flavonoid glycosides were deglycosylated by *β*-glucosidase in a cell free extract from a human small intestine (Day et al., 1998). These contradictory or differing results on the absorption of flavonoid glycosides calls attention to our understanding of the absorption of flavonoids from plant food. There is little knowledge on the relationship between structure and intestinal absorption, although flavonoid glycosides seem to be absorbed from the intestine in a native form or metabolites by intestinal bacteria. Efficiency of the absorption of flavonoids in the intestinal tract may depend on their individual structures.

In plasma, eriocitrin metabolites were mainly detected with eriodictyol, homoeriodictyol, and hesperetin in a conjugated form. The metabolites of both nonconjugates and conjugates were detected mainly in renal excreted urine for 24 h. Eriodictyol, homoeriodictyol, and hesperetin seemed to be the main eriocitrin metabolites in vivo. Naringin (naringenin 7-*O*-*β*-rhamnoglucoside), which is a flavanone glycoside in citrus fruit and is abundant in grapefruit, has been examined in renal excretion following administration of grapefruit juice in humans using HPLC analysis. It was reported that naringenin and its glucuronides were detected in the excreted urine for 24 h and that naringenin glucuronides were detected in plasma samples (Fuhr and Kummert, 1995). Eriocitrin and naringin seem to be similar in the metabolic pathways in vivo because both the nonconjugate and conjugate of each aglycones (eriodictyol and naringenin) were detected in urine and plasma.

The UDP-glucuronyl transferase was found in the intestinal mucosa of both small and large intestines (Silva et al., 1998). Luteolin and luteolin 7-*O*-*β*-glucoside were reported to be subjected to glucuronidation in intestinal absorption from our absorption experiment with an everted intestine (Shimoi et al., 1998). It has been proposed that ingested flavonoids are subjected to glucuronidation in intestinal mucosa as the first step in metabolic conversion. Eriodictyol, which is metabolized from eriocitrin by intestinal bacteria and absorbed from the intestine, seems to be subjected to glucuronidation intestinal absorption of intestine. The free metabolites were detected in urine (Figure 2) but were not detected in plasma (Figure 1). This suggests that the conjugated metabolites were metabolized in the kidneys and that the glucuronic acid and/or sulfate of them was released and the free metabolites were excreted in urine. It is generally believed that the liver is the main tissue for the metabolism of flavonoids (Piskula and Terao, 1998), includes methylation of hydroxy groups as well as conjugation with glucuronic acid and/or sulfuric acid (Terao, 1999). The *o*-dihydroxy structure at the 3′ and 4′ positions in the B ring may be the obvious target site for eriocitrin. The *O*-methylation seems to occur at the 3′ and 4′ positions of the B ring in the process of metabolic conversion of eriodictyol. We estimated that eriodictyol was metabolized to homoeriodictyol and hesperetin by methylation in the liver. It is suggested that the *o*-dihydroxyl structure in the B ring is involved in antioxidative activity. *O*-Methylation seems to be responsible for the loss of antioxidant activity.

We showed that the plasma following administration of eriocitrin exhibits a susceptibility to lipid peroxidation (Table 1) and that the antioxidants such as α-tocopherol,

ascorbic acid, uric acid, and bilirubin in the plasma did not influence the activity because the content of the antioxidants in rat plasma was not different (Table 2). Eriocitrin metabolites in plasma seem to exhibit the antioxidative activity of lipid peroxidation induced by AAPH and contribute to antioxidative activity. In the plasma of 4.0 h following administration of eriocitrin, conjugated and/or free of eriocitrin metabolites such as eriodictyol, homoeriodictyol, hesperetin, and 3,4-DHCA were detected (Figure 1). These metabolites in plasma suggest participation in antioxidative activity (Table 1). Furthermore, the plasma of 0.5, 1.0, and 2.0 h following administration of eriocitrin exhibited the same effect even though these metabolites were not detected. However, other eriocitrin metabolites of two unknown peaks, detected by HPLC analysis in experiments with an everted intestine, in their plasma may have participated in the activity, but it seems that there is a need for future examination.

We showed that free eriodictyol was detected in renal excreted urine (Figure 2). Eriodictyol, which has an *o*-dihydroxyl structure, was reported to have the highest antioxidative activity among the eriocitrin metabolites or flavonoids in lemon (Miyake et al., 1997b, 1998). Eriocitrin metabolites such as eriodictyol having an *o*-dihydroxyl structure seem to contribute to antioxidant activity in vivo following oral administration of eriocitrin. Eriodictyol seems to be a useful antioxidant and expresses an antioxidative activity in vivo such as the bladder or kidney, although it needs to be examined in the future. We calculated the absorption ratio of eriocitrin to be 5.68 ± 0.32% from eriocitrin metabolites in urine, but eriocitrin seemed to be metabolized into other compounds that we did not analyze for. Therefore, this absorption ratio estimate may be lower that the actual amount.

Various flavonoids are present mainly as flavonoid glycosides in our daily diets such as fruits, beans, and vegetables. Therefore, much attention has been focused on how glycosides are absorbed and whether absorbed glycosides can show antioxidant activity. The absorption pathway of flavonoid glycosides from the intestine may be roughly classified into two groups. One is hydrolyzed by β-glucosidase of the intestinal epithelium as luteolin 7-*O*-β-glucoside. The other requires intestinal microbacteria for the hydrolysis as rutin. In this paper we paid attention to eriocitrin of antioxidant in lemon fruit. Eriocitrin suggested to be mainly absorbed with the aglycone after digestion of intestinal bacteria and metabolized with glucuronidation, sulfation, and/or methylation. As the plasma of eriocitrin administered rats showed antioxidant activity, conjugated metabolites seem to possess antioxidant activity. The unknown peaks for metabolites of eriocitrin were detected in the serosal side of rat everted intestine and in the rat plasma after administration of eriocitrin. Further investigation on the metabolic mechanism and antioxidative activity of unknown peaks are required.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; 3,4-DHCA, 3,4-dihydroxyhydrocinnamic acid; MDA, malondialdehyde; LC−MS, liquid chromatography−mass spectrometry; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substance.

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