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Metabolism of Grape Seed Polyphenol in the Rat

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The metabolism of grape seed polyphenol (GSP) has been investigated in rats by high-performance liquid chromatography analysis of the serum and urinary concentrations of the GSP metabolites (+)-catechin (CT), (−)-epicatechin (EC), 3′-O-methyl-(+)-catechin, and 3′-O-methyl-(−)-epicatechin. The serum concentration of these four metabolites reached a maximum 3 h after the oral administration of GSP. The urinary excretion of these GSP metabolites accounted for 0.254% (w/w) of the administered dose of GSP (1.0 g/kg), and the majority of these metabolites were excreted within 25 h of oral administration. The serum concentration and urinary excretion of these metabolites were also compared after the oral administration of different GSP monomers (gallic acid, CT, and EC), normal GSP, and the high molecular weight components of GSP (GSPH). No metabolites were detected in the serum of rats given GSPH. The urinary percentage excretion of the GSP metabolites derived from the respective monomers (CT or EC) did not vary with the administration of different substances (CT or EC, GSP, or GSPH). Taken together, these results suggest that only the monomers of GSP are absorbed and metabolized.

KEYWORDS: Grape seed polyphenol; metabolism; rat; (+)-catechin; (−)-epicatechin; 3′-O-methyl-(+)-catechin; 3′-O-methyl-(−)-epicatechin

INTRODUCTION

Grape seed polyphenol (GSP) is a condensed tannin and has a complicated composition (*1*). The main constituent of GSP is proanthocyanidin, but it can contain some galloylated derivatives (*1*−*3*). The chemical structure of proanthocyanidin is shown in **Figure 1**. GSP has strong antioxidative properties both in vitro (*4*−*6*) and in vivo (*7*−*10*). Several beneficial physiological properties of GSP have been reported to date, including protection against radioactive or ultraviolet rays (*11*, *12*), chemoprevention (*13*, *14*), protection of cardiovascular system (*15*−*17*), anticancer effects (*18*−*21*), prevention of cataract formation (*22*), and cholesterol-lowering effects (*23*, *24*).

No toxicity has been reported for GSP (*25*−*28*), although it does interact with some types of protein (*29*−*31*). Donovan et al. (*32*) have reported that the proanthocyanidin component of GSP is not bioavailable in the rat. They also reported that only four metabolites, (+)-catechin (CT), (−)-epicatechin (EC), 3′-O-methyl-(+)-catechin (3′-OMCT), and 3′-O-methyl-(−)-epicatechin (3′-OMEC), are detected in serum and urine in the rat after the administration of GSP (*32*). The chemical structure of these four metabolites is shown in **Figure 2**. The metabolism of GSP as a whole, however, has not been clearly elucidated.

Thus, to analyze the metabolism of GSP in the rat in more detail, we have compared the metabolism of different components of GSP [GSP monomers, normal GSP, and the high molecular weight fraction of GSP (GSPH)] in the rat. In addition, we have investigated whether only GSP monomers or both monomers and the high molecular weight components of GSP are metabolized and utilized in vivo.

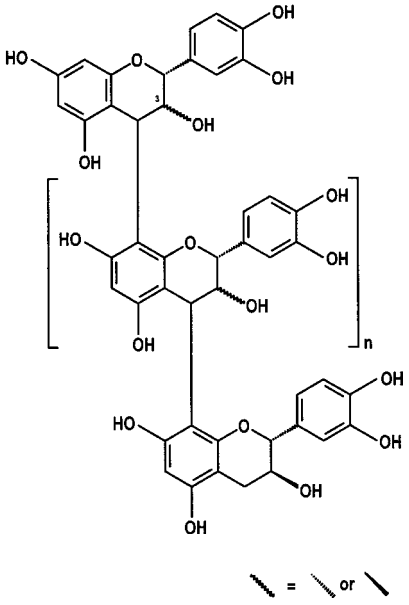

Figure 1. Chemical structure of proanthocyanidin.

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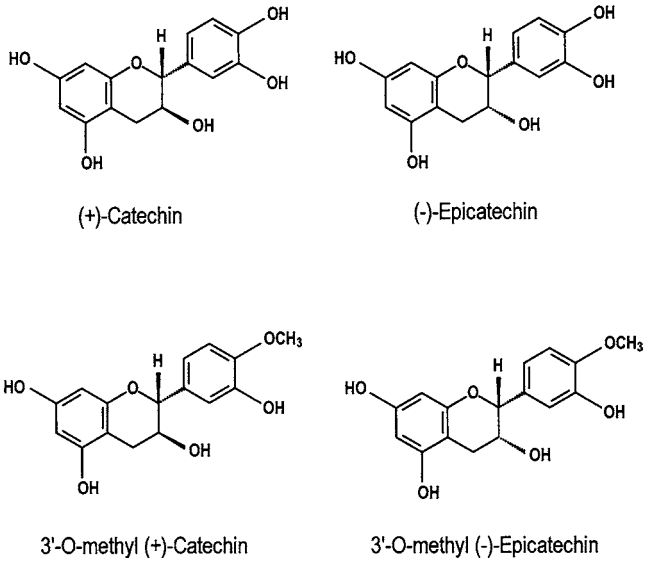

Figure 2. Chemical structures of GSP metabolites.

MATERIALS AND METHODS

Materials and Instruments. CT, EC, procyanidin B1 (ProB1), procyanidin B2 (ProB2), and procyanidin C1 (ProC1) for high-performance liquid chromatography (HPLC) analysis were purchased from Funakoshi (Tokyo, Japan). Gallic acid (GA), pyrogallol (PY), resorcinol (RE), and flavone (2-phenyl-4*H*-1-benzopyran-4-one, as an internal standard) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-O-Methyl gallic acid (4-OMGA) was purchased from Apin Chemicals Ltd. (Abingdon, U.K.). 3′-OMCT, 3′-OMEC, 4′-O-methyl-(+)-catechin (4′-OMCT), and 4′-O-methyl-(−)-epicatechin (4′-OMEC), synthesized by the method of Donovan et al. (*33*), was obtained from Wako Pure Chemical Industries, Ltd. The purity of these four compounds (determined by HPLC) was 99.2 (3′-OMCT), 98.6 (3′-OMEC), 99.5 (4′-OMCT), and 98.0% (4′-OMEC). Ion-exchanged and redistilled water was used throughout the experiments. HPLC-grade acetonitrile, ethyl acetate, *n*-hexane, and methanol were used for analyses. Other reagents were also analytical grade.

β-Glucuronidase (EC 3.2.1.31 from *Escherichia coli*, Type X-A, 100 000 units) and sulfatase (EC 3.1.6.1 from *Abalone entrails*, Type VIII, 1 000 units) were purchased from Sigma Chemical Co. (St. Louis, MO); tannase (EC 3.1.1.20, from *Aspergillus orizae*, 50 000 units) was from Wako Pure Chemical Industries. Sephadex LH-20 was purchased from Amersham Biosciences Corporation (Piscataway, NJ), and Sep-pak plus C_{18} (360 mg) and Sep-pak Envi C_{18} (840 mg) mini cartridge columns were from Waters Corporation (Milford, MA). The HPLC columns STR ODS-II (4.6 mm i.d. × 250 mm) and Develosil 300 ODS-HG-5 (4.6 mm i.d. × 250 mm) were purchased from Shinwa Chemical Industries, Ltd. (Kyoto, Japan) and Nomura Chemical Co., Ltd. (Seto, Japan), respectively.

Substances for Oral Administration. GA, CT (purity 98% by HPLC), and EC (purity 90% by HPLC) for oral administration to rats were purchased from Wako Pure Chemical Industries Ltd., Sigma Chemicals Co., Ltd., and Aldrich Chemical Co., Ltd. (Milwaukee, WI), respectively. GSP for oral administration to rats was purchased from Tokiwa Phytochemical Co., Ltd. (Sakura, Japan). The content of proanthocyanidin in GSP was more than 99%, according to the manufacturer's details. It was determined as 102.1 ± 1.6 w/w % by the vanillin−HCl assay (*34*) using CT as the standard [means ± standard deviation (SD) for five trials]. The composition of the low molecular weight compounds in GSP, determined by HPLC (*35*), was as follows: GA, 0.14 ± 0.01 w/w %; CT, 3.24 ± 0.15 w/w %; EC, 2.06 ± 0.12 w/w %; ProB1, 1.54 ± 0.10 w/w %; ProB2, 1.35 ± 0.05 w/w %; and ProC1, 1.26 ± 0.52 w/w %.

The GSPH was prepared by the method of Kants et al. (*36*) with a few modifications. In brief, six Sephadex LH-20 columns were individually prepared as follows. Thirty grams of Sephadex LH-20 suspended in water was placed in a glass column (20 mm i.d. × 500 mm) with a cotton plug, and the column was washed with 200 mL of water. A solution of 0.2 g of GSP in 5 mL of water was applied to the column, which was then washed with 200 mL of water, followed by 500 mL of a solution of water/methanol/acetic acid (400:600:2, v/v/v). Then, the column was eluted with 900 mL of a mixture of methanol:acetic acid (1000:2, v/v), followed by 100 mL of a mixture of acetone:acetic acid (1000:2, v/v). The fractions from these two elutions were collected (the GSPH fraction) from each Sephadex LH-20 column and evaporated to dryness. Each residue was resuspended in 100 mL of water, and 5 mL of this suspension was applied to a Sep-pak Envi C_{18} mini cartridge column that had been prewashed with 10 mL of methanol followed by 10 mL of water. The Sep-pak Envi C_{18} mini cartridge column was washed with 10 mL of water and eluted with 10 mL of methanol. Each eluate was collected, evaporated to dryness, and dried in a desiccator overnight to obtain a residue of GSPH. The recovery of GSPH from GSP was 35.0 w/w %. The content of proanthocyanidin in GSPH was 101.6 ± 0.6 w/w % (means ± SD for five trials), determined as CT by the vanillin−HCl assay (*34*). The composition of the low molecular compounds in GSPH, determined by HPLC (*35*), was as follows: GA, 0%; CT, 0.24 w/w %; EC, 0.20 w/w %; ProB1, 0.41 w/w %; ProB2, 0.39 w/w %; and ProC1, 1.12 w/w % (average of two trials). HPLC chromatograms of GSP (0.2 g/mL in methanol solution) and GSPH (derived from GSP, 0.2 g in a final volume of 1 mL of methanol) analyzed using a Develosil 300 ODS-HG-5 column are indicated in **Figure 3**.

A Shimadzu HPLC apparatus, Class-VP Series (Kyoto, Japan), equipped with system controller SCL-10AVP, pump LC-10ADVP, degasser DGU-14A, autoinjector SIL-10ADVP, column oven CTO-10AC, fluorescent detector RF-10AXL, and diode array detector (DAD) SPD-M10AVP, was used to analyze GSP and its metabolites. The temperature of the water bath attached to the rotary evaporator was set below 40 °C.

Animal Experiments. All procedures involving animals were conducted according to Japanese law (Bulletin of the Prime Minister's Office No. 6, March 1980) and guidelines established by the National Institute of Health Sciences. Male Wister rats (4 weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan) and kept in an air-conditioned room (23 ± 1 °C, 50−60% humidity) illuminated for 12 h a day (7:00 to 19:00). Rats weighing 286−304 and 259−296 g were used in experiments 1 and 2 and in experiments 3 and 4, respectively. Rats had free access to a normal F-2 diet (Funahashi Farm, Chiba, Japan) and water throughout the experiments. No GSP components were detected in the F-2 diet. The solution of GSP, GA, and CT used for oral administration was freshly prepared before use by dissolving 0.1−1.0 g of the appropriate compound in water and adjusting the volume to 10 mL. The solutions were administered to rats at a dose of 10 mL/kg body weight between 13:00 and 15:00 h.

In experiment 1, GSP was administered orally to rats at a dose of 1.0 g/kg. Each group consisted of 3−6 rats. Rats were anesthetized with diethyl ether, and blood was collected by heart puncture at 0.5, 1, 1.5, 2, 3, 4, 5.5, 7, and 20 h after oral administration. Serum was obtained by centrifugation of blood at 1500 rpm for 15 min at ambient temperature and stored below −20 °C until analysis. In experiment 2, GSP was administered orally to six rats at a dose 1.0 g/kg. Urine and feces were collected at 5.5, 24.5, and 54 h and 24.5 and 54 h after GSP administration, respectively. Collected urine was immediately filtered through no. 5A filter paper, adjusted to 50 mL after the addition of 100 mg of ascorbic acid, and stored below −20 °C until analysis. Collected feces were dried overnight at 40 °C, ground to a powder, and stored below −20 °C until analysis.

In experiment 3, GA, CT, and EC were administered orally at a dose of 0.3 g/kg; GSP was administered at doses of 0.1, 0.2, 0.5, and 1.0 g/kg; and GSPH was administered at a dose of 0.5 g/kg. Each experimental group consisted of 3−5 rats. Rats were treated as described in experiment 1. Blood was collected at 2.25 h after oral administration, and serum was obtained as described in experiment 1.

In experiment 4, CT and EC were administered orally at a dose of 0.3 g/kg, and GSP and GSPH were administered at a dose of 0.5 g/kg, respectively. Each group consisted of four rats. Urine was collected at 5.5, 24.5, and 54 h after oral administration and treated as described in experiment 2.

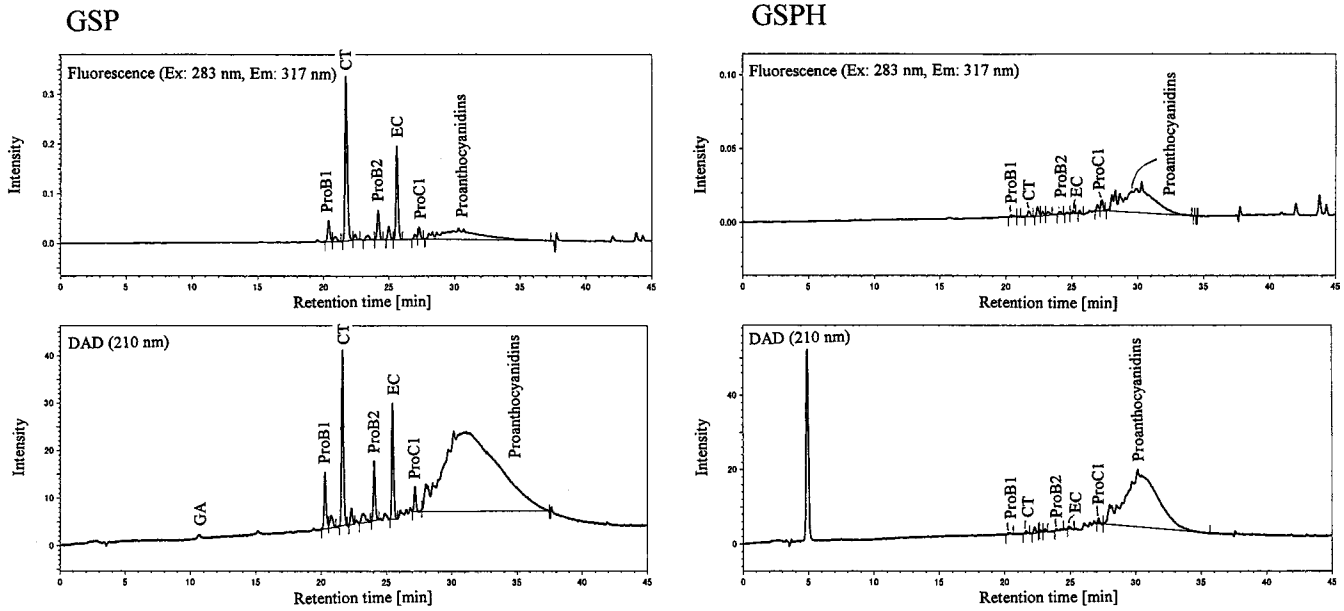

Figure 3. HPLC chromatograms of GSP and GSPH. GSP 0.2 g/mL and GSPH (derived from GSP 0.2 g; 0.2 g in a final volume of 1 mL) were analyzed by HPLC using a Develosil 300 ODS-HG-5 column. Conditions for HPLC are described in the text.

Analytical Methods. *Analysis of the Metabolites of GSP, GA, CT, EC, and GSPH in Serum and Urine.* Metabolites of GSP, GA, CT, EC, and GSPH in serum and urine were analyzed as follows. To 0.5 mL of serum or 0.5−2 mL of urine, 7 mL of 0.1 M acetate buffer (pH 6.8), 0.1 mL of 4% ascorbic acid solution (in water), 0.1 mL of 4% Na_2EDTA solution (in water), and 0.2 mL of 0.6 M $CaCl_2$ solution (in water) were added. For the determination of total metabolites, β-glucuronidase (1000 units for serum and 3000 units for urine) and sulfatase (25 units for serum and 75 units for urine) were added to the serum preparation, which was then incubated at 37 °C for 45 min. Enzymatic hydrolysis was stopped by the addition of 0.5 mL of 1 N HCl.

For the analysis of serum metabolites or GA, 4-OMGA, PY, and RE in urine, after the enzymatic hydrolysis step and the addition of flavone (23.6 nmol) as an internal standard, the metabolites were extracted three times with 10 mL of ethyl acetate. For the analysis of CT, EC, 3′-OMCT, and 3′-OMEC in urine, the hydrolysis reaction mixture was applied to a Sep-pak plus C_{18} mini cartridge column that had been prewashed with 10 mL of methanol followed by 10 mL of water after the addition of flavone (23.6 nmol) as an internal standard. The column was washed with 10 mL of water, and the metabolites were eluted with 10 mL of methanol. After evaporation of the ethyl acetate extracts or the methanol eluate to dryness, the residue was dissolved in 2 mL of methanol for analysis by HPLC. Free metabolites were analyzed in the same way but without the enzymatic hydrolysis step.

Analysis of GSP and GSP Metabolites in Feces. Fecal GSP samples were analyzed in triplicate. About 0.2 g of dried, ground feces was weighed exactly, and the lipids were extracted with 50 mL of *n*-hexane at ambient temperature for 24 h after 30 min of sonication. The residue was dried, and the GSP metabolites therein were extracted with 50 mL of methanol (24 h at ambient temperature after 30 min of sonication). The methanol extract was adjusted to 50 mL (MeOH soluble fraction), and 2 mL aliquots were evaporated to dryness. The GSP metabolites were analyzed as described above.

Total GSP content was analyzed by a previously reported method (*37*) with a few modifications. About 0.2 g of dried, ground feces was weighed exactly, and the lipids were extracted with 50 mL of *n*-hexane at ambient temperature for 24 h after 30 min of sonication. After the *n*-hexane was evaporated completely, 20 mL of 0.05 M citrate buffer (pH 5.5) containing tannase (2000 U) was added and the mixture was incubated at 30 °C for 20 h. Tannase was inactivated by the addition of 1 mL of 1.0 N HCl. After the addition of flavone (944.9 nmol) as an internal standard, the hydrolyzate was extracted three times with 20 mL of ethyl acetate. After evaporation to dryness, the residue was dissolved in methanol, and the volume was adjusted to 50 mL and analyzed by HPLC.

To calculate the amount of GSP, 1−50 mg of GSP was hydrolyzed with tannase (2000 U) at 30 °C for 20 h. After the addition of flavone (944.9 nmol) as an internal standard, the GA liberated from the GSP standard was extracted three times with ethyl acetate. After evaporation to dryness, the residue was dissolved in methanol, and the volume was adjusted to 50 mL and analyzed by HPLC. A calibration curve relating GSP to GA was derived on the basis of the GA liberated from the GSP standard. The fecal content of GSP was determined from the calibration curve and corrected by subtracting the control value (no tannase treatment).

HPLC. The HPLC conditions were as follows: column, STR ODS-II (for serum and urinary metabolite analysis) or Develosil 300 ODS-HG-5 (for analysis of fecal GSP and its metabolites); column oven temperature, 35 °C; mobile phases, (A) water/phosphoric acid 1000:1 (v/v), (B) acetonitrile/phosphoric acid 1000:1 (v/v/v); a gradient program, (B) 0% (0 min) → 15% (20 min) → 30% (25 min) → 100% (45−61 min) → 0% (62−77 min); fluorescent detector, Ex 283 nm, Em 317 nm for CT, EC, 3′-OMCT, 3′-OMEC, 4′-OMCT, 4′-OMEC, ProB1, ProB2, ProC1, and RE; DAD, wavelengths monitored, 210 nm for GA, 4-OMGA, PY, and flavone; injection volume, 10 μL.

Recovery Tests. Tests for the recovery of GSP and its metabolites from serum, urine, and feces were performed in five trials using serum, urine, and dried feces samples from a control rat. Appropriate amounts of the GSP metabolites and related compounds (CT, EC, 3′-OMCT, 4′-OMCT, 3′-OMEC, 4′-OMEC, ProB1, ProB2, ProC1, GA, PY, RE, and 4-OMGA) plus flavone (as an internal standard) were added to 0.5 mL of serum, 2 mL of urine, or 2 mL of the MeOH soluble fraction derived from 0.2 g of dried feces. The GSP metabolites and related compounds were analyzed as described above without enzymatic hydrolysis.

To test for the recovery of GSP, 10 mg of GSP was added to feces samples after they had been defatted by *n*-hexane. Samples were treated and analyzed as described above, and the recoveries were calculated.

Statistics. Data are expressed as means ± standard error of the means (SEM). Dose−response was examined by linear regression analysis after one way analysis of variance (ANOVA). Three way ANOVA (completely randomized design) was performed for the results of experiment 4. Each factor is defined as follows. Factor A was defined as the collecting period of urine after oral administration: A1, 0−4.5 h; A2, 4.5−25 h; and A3, 25−49 h. Factor B was defined as the administered substance: B1, monomer (CT and EC); B2, GSP; and B3, GSPH. Factor C was defined as the percentage excretion (w/w %)

Table 1. Relative Retention Times and Detection Limits of Polyphenols by a STR ODS-II Column

polyphenol[a]	molecular weight	relative retention time (flavone = 1.00) (determined by DAD)	detection limit (S/N = 3) (pmol/mL) fluorescence (Ex 283 nm, Em 317 nm)	detection limit (S/N = 3) (pmol/mL) ultraviolet (210 nm except 260 nm for EA)	linear range of calibration curve (pmol/mL) (r^2) fluorescence (Ex 283 nm, Em 317 nm)	linear range of calibration curve (pmol/mL) (r^2) ultraviolet (210 nm)
CT	290.3	0.600	8.04	80.4	8.04–8044 (0.995)	80.4–40217 (0.998)
EC	290.3	0.684	5.16	51.6	5.16–10317 (0.996)	51.6–51585 (0.999)
3′-OMCT	304.3	0.710	4.49	44.9	4.49–8988 (0.995)	44.9–44939 (0.998)
4′-OMCT	304.3	0.735	5.20	52.0	5.20–10409 (0.996)	52.0–52046 (0.998)
3′-OMEC	304.3	0.741	4.62	46.2	4.62–9243 (0.994)	46.2–46213 (0.999)
4′-OMEC	304.3	0.759	5.93	59.3	5.93–11867 (0.999)	59.3–59335 (0.999)
ProB1	578.5	0.546	37.6	83.4	37.6–3730 (0.993)	83.4–7468 (0.998)
ProB2	578.5	0.645	23.8	47.5	23.8–4750 (0.994)	47.6–9510 (1.000)
ProC1	866.8	0.698	12.9	51.4	12.9–2570 (0.995)	37.3–7468 (1.000)
GA	170.1	0.324		238.4		238.4–119193 (0.997)
PY	126.1	0.294		294.4		294.4–147204 (0.998)
RE	110.1	0.441	46.1	461.4	46.1–19368 (0.995)	461.4–230679 (0.998)
4-OMGA	184.1	0.527		96.8		96.8–96838 (0.998)
flavone (IS)	222.2	1.000				

[a] Abbreviations are as follows: 3′-OMCT, 3′-O-methyl catechin; 3′-OMEC, 3′-O-methyl epicatechin; 4′-OMEC, 4′-O-methyl epicatechin. Conditions for HPLC are described in the text.

Table 2. Recoveries of the Components of GSP, Its Metabolites, and Their Related Compounds

related compds[b]	added amounts	recoveries (%)[a] serum (0.5 mL)[c]	recoveries (%)[a] urine (2 mL/ 50 mL for 1 day)[c]	dried feces (0.2 g) methanol soluble fraction[c]	dried feces (0.2 g) total[c]
CT	8.04 nmol	80.0 ± 5.7	88.8 ± 3.9	86.9 ± 6.0	not determined
EC	10.3 nmol	73.5 ± 6.0	90.1 ± 4.8	78.4 ± 3.1	not determined
3′-OMCT	8.99 nmol	82.0 ± 4.2	102.3 ± 10.0	89.5 ± 5.7	not determined
4′-OMCT	10.4 nmol	83.0 ± 5.7	97.9 ± 6.2	89.8 ± 7.1	not determined
3′-OMEC	9.24 nmol	75.1 ± 6.7	91.3 ± 2.4	81.8 ± 6.5	not determined
4′-OMEC	11.9 nmol	77.3 ± 6.0	110.8 ± 5.2	83.3 ± 4.3	not determined
ProB1	18.7 nmol	50.4 ± 5.2	77.8 ± 4.8	57.2 ± 1.6	not determined
ProB2	23.8 nmol	69.1 ± 5.8	85.2 ± 4.4	76.1 ± 1.8	not determined
ProC1	12.8 nmol	40.4 ± 2.1	74.2 ± 2.1	52.2 ± 1.2	not determined
GA	23.8 nmol	95.4 ± 5.0	96.7 ± 4.5	94.9 ± 3.1	not determined
PY	18.7 nmol	94.5 ± 5.2	100.7 ± 5.8	95.1 ± 2.4	not determined
RE	46.1 nmol	95.9 ± 5.9	103.9 ± 5.0	93.5 ± 3.3	not determined
4-OMGA	19.4 nmol	103.2 ± 4.7	100.9 ± 4.5	100.9 ± 4.1	not determined
GSP	10 mg	not determined	not determined	not determined	32.5 ± 11.7

[a] Data are means ± SD for five trials. Conditions for HPLC are described in the legend of Table 1. [b] Abbreviations are listed in the legend of Table 1. [c] Recovery tests of GSP, GSP metabolites, and related compounds from serum, urine, and the dried feces were performed by the methods described in the text without enzymatic hydrolysis.

of each metabolite divided by the net amount of the corresponding GSP monomer in each administered substance [the percentage excretion of CT or 3′-OMCT was divided by the net amount of CT in GSP (3.24%) or GSPH (0.24%), and the percentage excretion of EC or 3′-OMEC was divided by the net amount of EC in GSP (2.06%) or GSPH (0.20%)]: C1, CT; C2, 3′-OMCT; C3, EC; and C4, 3′-OMEC. Probability values of less than 0.05 were considered statistically significant.

RESULTS

Analytical Methods. The retention times of the GSP metabolites and related compounds (relative to that of the internal standard flavone, which was set at 1.00) and their detection limits are shown in **Table 1**. Each compound was resolved completely by HPLC.

The recovery of the GSP metabolites and related compounds from serum, urine, and feces is shown in **Table 2**. The recovery of ProB1, ProB2, and ProC1 from serum, urine, and dried feces was relatively low (40.4−69.1% from serum, 74.2−85.2% from urine, and 52.2−76.1% from dried feces). For the other compounds, however, the recovery from serum, urine, and dried feces was 73.5−103.2, 88.8−103.9, and 78.4−100.9%, respectively. The recovery of GSP from feces is also shown in **Table 2**. The recovery of GSP was low (32.5%), and the variance was large.

Metabolism of GSP in the Rat. Figure 4 shows the concentrations of GSP metabolites in serum as a function of time. Only CT, EC, 3′-OMCT, and 3′-OMEC were detected in serum. Most of the GSP metabolites in serum were conjugated with glucuronide or sulfate, and only trace amounts of the free forms were detected. The concentrations of CT, EC, 3′-OMCT, and 3′-OMEC reached maximal values in serum 3 h after oral administration. These metabolites were almost completely cleared from serum 20 h after the administration of GSP. **Figure 5** shows the urinary concentrations of GSP metabolites with or without hydrolysis by β-glucuronidase and sulfatase as a function of time. CT, EC, 3′-OMCT, and 3′-OMEC were detected in urine. More than 80% of the GSP metabolites detected was excreted within 25 h of GSP administration: CT,

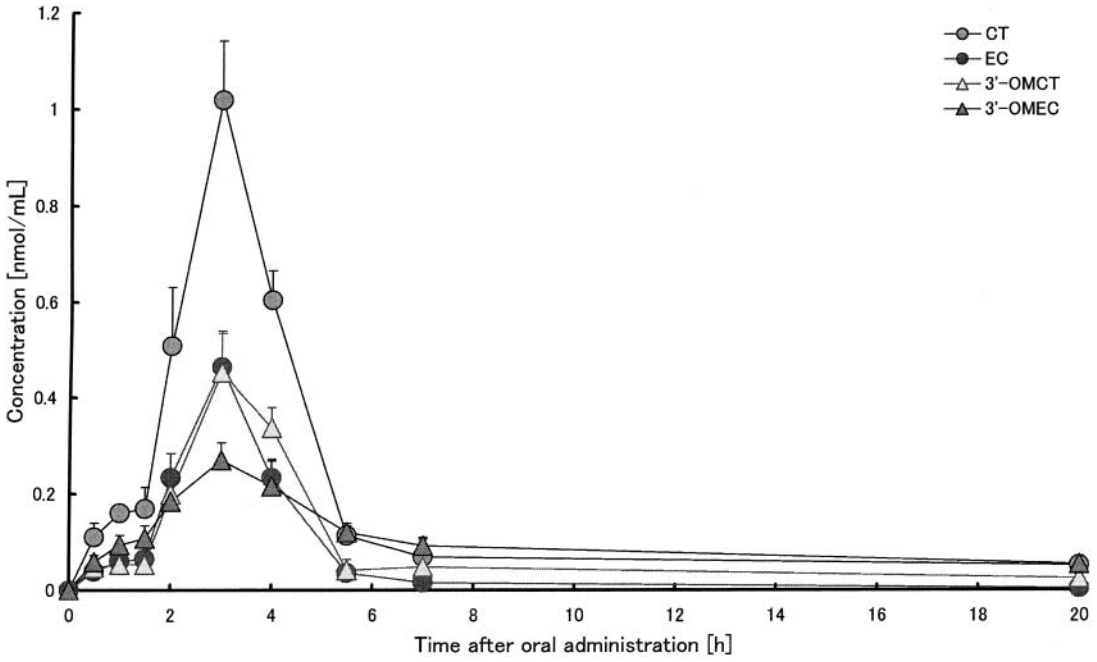

Figure 4. Serum concentrations of GSP metabolites as a function of time (GSP 1.0 g/kg). Mean values are shown; vertical lines indicate the SEM for 3–6 rats.

Table 3. Percentage Excretion of GSP and Its Metabolites in Urine and Feces (GSP 1.0 g/kg)

	percentage excretion[a] (w/w % of total)						
	in urine (h)				in feces (h)		
compd	0–4.5	4.5–25	25–49	0–49	0–25	25–49	0–49
GSP					20.9 ± 3.7	1.6 ± 0.9	22.5 ± +3.9
CT	0.027 ± 0.007	0.024 ± 0.006	0.005 ± 0.004	0.055 ± 0.014	ND[b]	ND	ND
EC	0.019 ± 0.005	0.017 ± 0.005	0.004 ± 0.003	0.040 ± 0.011	ND	ND	ND
3′-OMCT	0.024 ± 0.006	0.053 ± 0.009	0.011 ± 0.005	0.088 ± 0.013	ND	ND	ND
3′-OMEC	0.019 ± 0.003	0.038 ± 0.008	0.015 ± 0.003	0.071 ± 0.010	ND	ND	ND

[a] Data are means ± SEM for six rats. [b] ND, not detected.

84.9%; EC, 90.2%; 3′-OMCT, 87.7%; and 3′-OMEC, 79.8%. The percentages of the free forms of urinary GSP metabolites excreted within 49 h of GSP administration were 34.2% for CT, 27.5% for EC, 29.4% for 3′-OMCT, and 49.1% for 3′-OMEC. In contrast to urine, no GSP metabolites were detected in feces.

The percentage excretion (w/w % of total) of GSP and its metabolites in urine and feces is shown in **Table 3**. Excretion of the GSP metabolites in urine was rapid as follows: 26.3−48.5% of the GSP metabolites was excreted within 4.5 h, and 79.7−91.7% was excreted within 25 h of GSP administration. Within 49 h of oral administration, GSP was excreted in urine as CT (0.055%), EC (0.040%), 3′-OMCT (0.088%), and 3′-OMEC (0.071%), accounting for 0.254% of the administered dose. In feces, 20.9% of the administered dose of GSP was excreted as GSP within 25 h, and 22.5% was excreted within 49 h.

Comparison of the Metabolism of GSP Monomer, GSP, and GSPH in the Rat. Figure 6 shows the concentrations of GSP metabolites in rat serum 2.25 h after the oral administration of CT, EC, or GA at a dose of 0.3 g/kg, GSP at doses of 0.01−1.0 g/kg, or GSPH at a dose of 0.5 g/kg. Serum CT and 3′-OMCT were detected after CT administration; serum EC and 3′-OMEC were detected after EC administration; and serum GA and 4-OMGA were detected after GA administration. Serum CT, EC, 3′-OMCT, and 3′-OMEC were detected after the administration of GSP, and the concentrations of these four metabolites increased dose-dependently, as shown in **Table 4**. No metabolites were detected in the serum of rats given GSPH. The percentages of the free metabolite forms were 23.2% for GA and 46.8% for 4-OMGA in the serum of rats given GA, whereas more than 96% of the detected metabolites was conjugated with glucuronide or sulfate in the serum of rats given CT or EC. Free metabolite forms were not detected in the serum of rats given GSP.

Figure 7 shows the urinary concentrations of the metabolites in rats given CT or EC at a dose of 0.3 g/kg and GSP or GSPH at a dose of 0.5 g/kg with or without hydrolysis by β-glucuronidase and sulfatase as a function of time. Urinary CT and 3′-OMCT were detected after CT administration; urinary EC and 3′-OMEC were detected after EC administration; and urinary CT, EC, 3′-OMCT, and 3′-OMEC were detected after GSP administration. Only trace amounts of CT, EC, 3′-OMCT, and 3′-OMEC were detected in the urine of rats given GSPH, as compared with the other groups. More than 78.5% of the metabolites detected was excreted within 25 h of oral administration: 98.3% of CT and 98.7% of 3′-OMCT in rats given CT; 97.6% of EC and 96.3% of 3′-OMEC in rats given EC; 96.3% of CT, 97.1% of EC, 97.9% of 3′-OMCT, and 92.7% of 3′-OMEC in rats given GSP; and 100% of CT, 92.6% of EC, 98.9% of 3′-OMCT, and 78.5% of 3′-OMEC in rats given GSPH. The percentages of the free metabolite forms excreted in urine within 49 h of oral administration were 6.1% for CT and 10.0% for 3′-OMCT in rats given CT; 11.2% for EC and

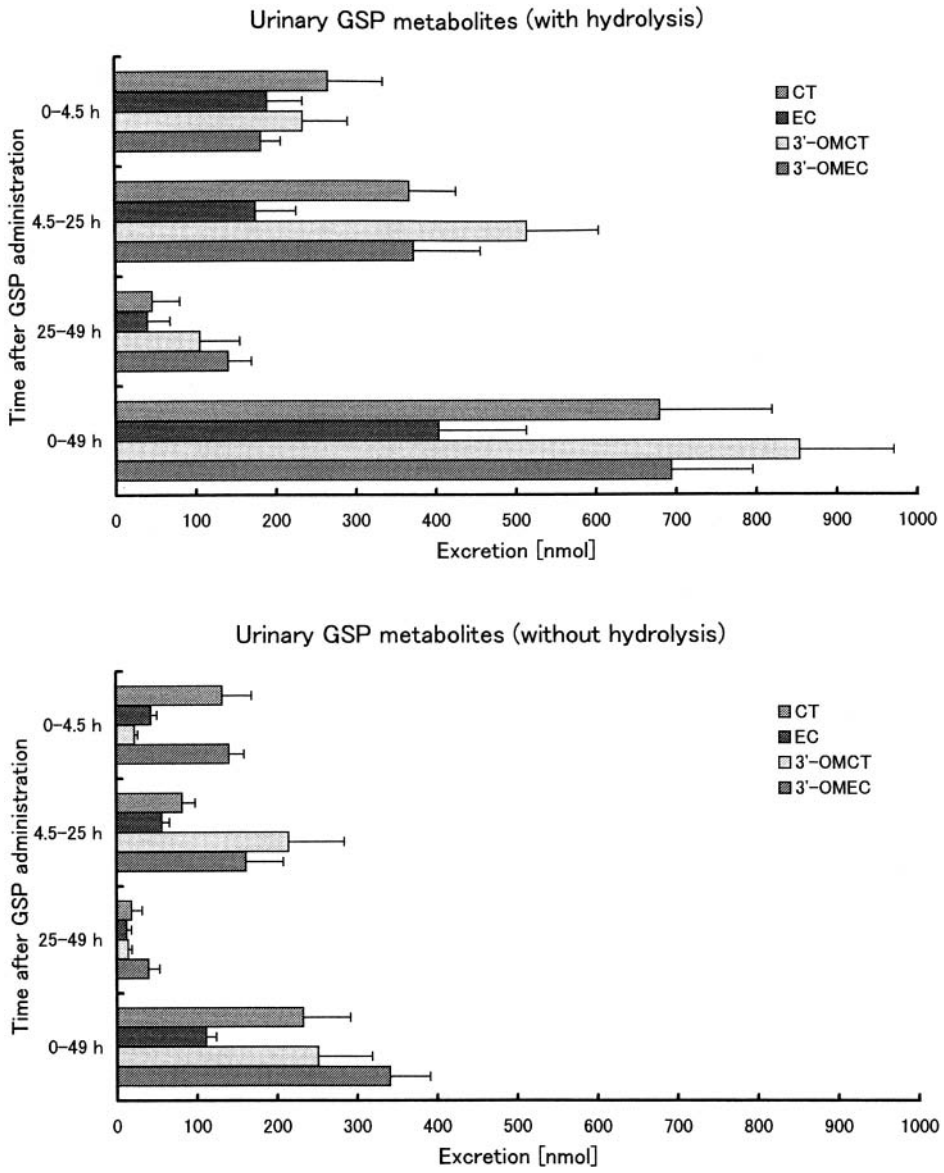

Figure 5. Urinary excretion of GSP metabolites in rats given GSP as a function of time. Mean values are shown, and horizontal lines indicate the SEM for six rats.

12.4% in rats given EC; 13.0% for CT, 12.3% for EC, 6.6% for 3′-OMCT, and 16.5% for 3′-OMEC in rats given GSP; 13.2% for EC and 0% for CT, 3′-OMCT, and 3′-OMEC in rats given GSPH.

The percentage excretion (w/w % of total) of the metabolites in urine in rats administered CT or EC at a dose of 0.3 g/kg and GSP or GSPH at a dose of 0.5 g/kg is shown in **Table 5**. Excretion of the urinary metabolites was rapid as follows: after CT administration, 22.8−37.4% of the detected metabolites was excreted within 4.5 h, and 98.2−98.6% was excreted within 25 h; after EC administration, 53.7−68.5% of the detected metabolites was excreted within 4.5 h, and 97.6−98.3% was excreted within 25 h; after GSP administration, 34.9−53.8% of the detected metabolites was excreted within 4.5 h, and 91.3−97.9% was excreted within 25 h; after GSPH administration, 34.7−100% of the metabolites was excreted within 4.5 h, and 79.5−100% was excreted within 25 h. Within 49 h of oral administration, CT was excreted in urine as CT (3.25%) and 3′-OMCT (1.66%), accounting for 4.91% of the administered CT dose; EC was excreted in urine as EC (2.74%) and 3′-OMEC (1.00%), accounting for 3.74% of the administered EC dose; GSP was excreted in urine as CT (0.090%), EC (0.064%), 3′-OMCT (0.102%), and 3′-OMEC (0.025%), accounting for 0.281% of the administered GSP dose; and GSPH was excreted in urine as CT (0.002%), EC (0.006%), 3′-OMCT (0.004%), and 3′-OMEC (0.005%), accounting for 0.017% of the administered GSPH dose.

Analysis by three way ANOVA (completely randomized design) for factors A (the collecting period of urine), B (the administered substance), and C [the percentage excretion (w/w %) of each metabolites divided by the net amount of the correspondent monomer in each administered substances] indicated that there was no statistical difference in factor B ($p = 0.06772$), as shown in **Table 6**.

DISCUSSION

Although various studies have been conducted on the physiological properties of GSP (*7*−*24*), it remains unknown which components of GSP, namely, monomers, oligomers, or polymers, are available and beneficial in vivo. The monomers CT and EC are found in GSP; they are constituents of the various kinds of proanthocyanidin that comprise the main component of GSP. There have been several studies on the

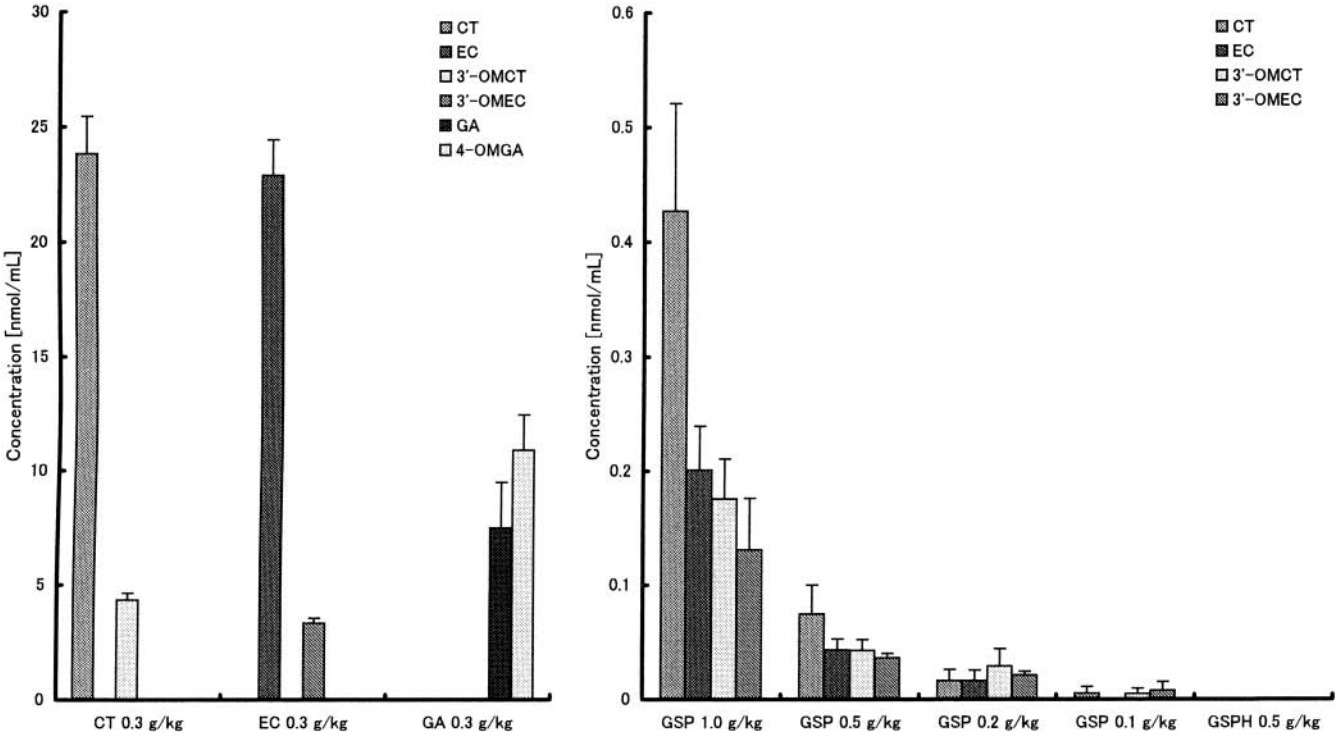

Figure 6. Serum concentration of GSP metabolites in rats given CT, EC, GA, GSP, or GSPH at 2.25 h after oral administration. Values are means, and each vertical line indicates SEM for 3–6 rats.

Table 4. Dose–Response of Serum GSP Metabolite Concentration

metabolites	dose–response[a]	r^2
CT	$p < 0.005$	0.4918
EC	$p < 0.001$	0.5489
3′-OMCT	$p < 0.005$	0.5122
3′-OMEC	$p < 0.02$	0.3354

[a] Dose–response was analyzed by linear regression analysis.

metabolism of CT or EC and on 3′-OMCT and 3′-OMEC, which are the respective metabolites of CT and EC (*38*−*42*). It has been proposed that methylation at the 3′-OH of CT or EC by catechol-O-methyl transferase (EC 2.1.1.6) occurs in the liver or the intestine to produce 3′-OMCT or 3′-OMEC, respectively (*43*).

So far, there has been only one paper by Donovan et al. (*32*) on the metabolism of GSP, but the time course of the GSP metabolites in serum and urine has not been clearly elucidated. More detailed data about the metabolism of GSP are needed to elucidate the physiological properties of GSP. We therefore investigated the metabolism of GSP in the rat precisely as a function of time for the first time after the oral administration of a single dose of 1.0 g/kg GSP. In addition, to investigate which components of GSP contribute to the bioavailability of GSP, we compared the metabolism of GSP monomers (GA, CT, or EC), normal GSP, and the GSPH after their oral administration in the rat by determining the serum concentrations and urinary excretion of the various metabolites.

Before carrying out the animal experiments, we examined different methods for analyzing the metabolites. The detection limits of CT, EC, 3′-OMCT, 3′-OMEC, 4′-OMCT, 4′-OMEC, ProB1, ProB2, ProC1, and RE achieved using the fluorescence detector were 2−10 times lower than those achieved by DAD at 210 nm (**Table 1**); thus, the analysis of these compounds was done using the fluorescence detector. When an ethyl acetate extraction was used for the analysis of urine without cleanup on a Sep-pak plus C_{18} mini cartridge column, the incidence of interfering peaks on the HPLC chromatogram increased and it became difficult to determine the content of CT, EC, 3′-OMCT, 3′-OMEC, 4′-OMCT, and 4′-OMEC (data not shown). However, the recoveries of GA, 4-OMGA, PY, and RE were only 0−36.3% when the urine sample was cleaned up by the Sep-pak plus C_{18} mini cartridge column. Thus, urine samples were cleaned up by Sep-pak plus C_{18} mini cartridge column for the analysis of CT, EC, 3′-OMCT, 3′-OMEC, 4′-OMCT, and 4′-OMEC but were extracted by ethyl acetate for the analysis of GA, 4-OMGA, PY, and RE. The recoveries of ProB1, ProB2, and ProC1 were relatively low (40.4−69.1% from serum, 74.2−85.2% from urine, and 52.2−76.1% from dried feces); however, these compounds were not detected in serum, urine, and feces in rats given GSP or GSPH. As a whole, the recoveries of GSP metabolites and their related compounds from serum, urine, and feces were relatively good (more than 73.5%). Therefore, we used these methods to analyze GSP and its metabolites in serum, urine, and feces in order to examine the metabolism of GSP in rats.

Only CT, EC, 3′-OMCT, and 3′-OMEC were detected in serum and urine (**Figures 4** and **5** and **Table 3**) as Donovan et al. (*32*) previously reported. Proanthocyanidin dimers (ProB1 and ProB2), trimers (ProC1), and the metabolites of GA (GA, 4-OMGA, PY, and RE) were not detected. The metabolism and excretion of CT, EC, 3′-OMCT, and 3′-OMEC seemed to be relatively fast: Serum concentrations of CT, EC, 3′-OMCT, and 3′-OMEC reached maximal values at 3 h and could barely be detected 20 h after GSP administration GSP (**Figure 4**). Donovan et al. (*32*) previously reported that the percentage of CT in methylated form in rat plasma tended to increase with time after the administration of diets containing CT or GSP; however, we did not observe this tendency (**Figure 4**). This apparent discrepancy might be due to differences in the method of administration: whereas Donovan et al. (*32*) administered GSP in the diet, we administered GSP in a single dose.

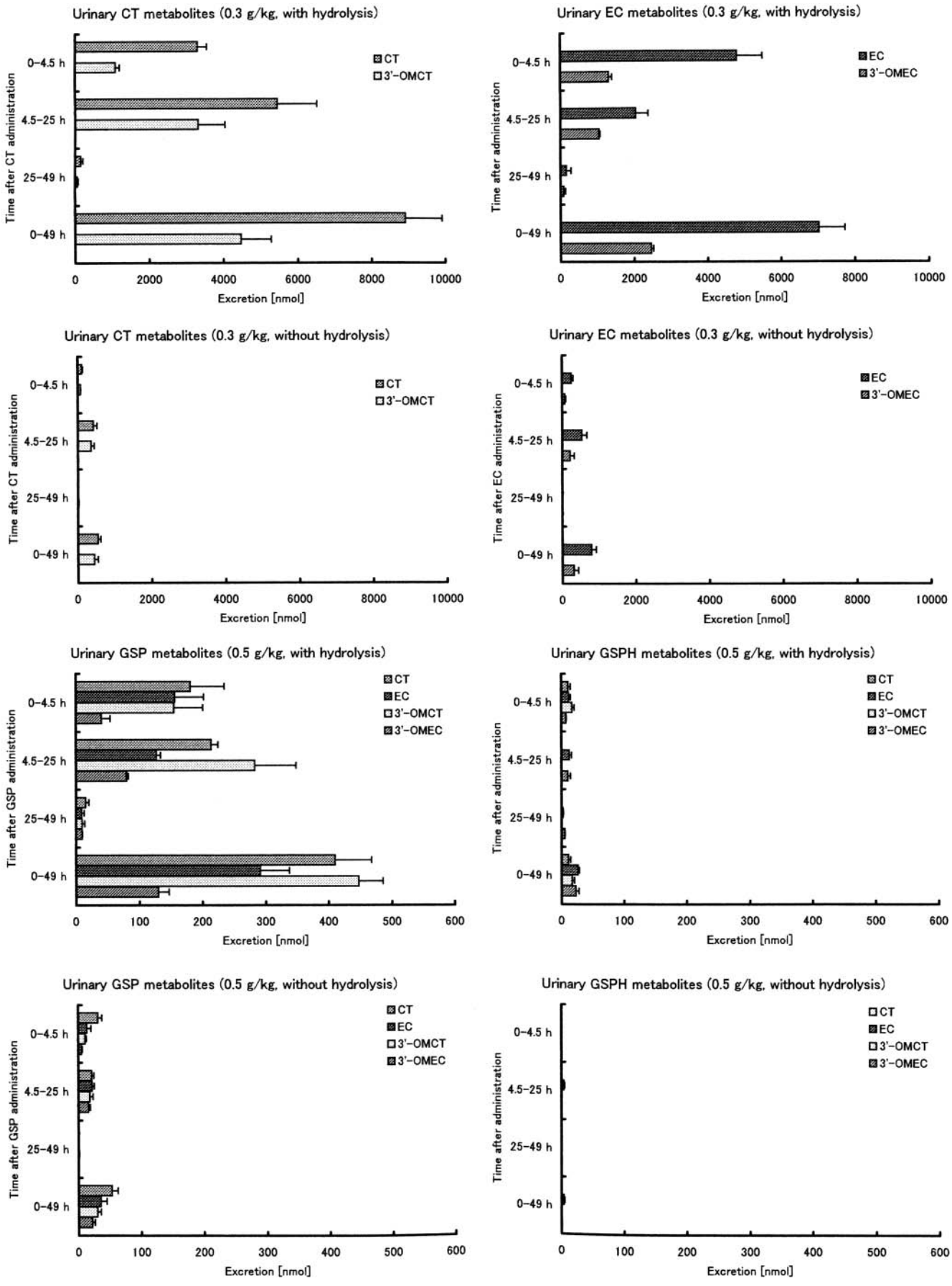

Figure 7. Urinary excretion of the metabolites in rats given CT, EC, GSP, or GSPH as a function of time. Mean values are shown, and horizontal lines indicate the SEM for four rats.

Table 5. Percentage Excretion of the Metabolites of EC, CT, GSP, and GSPH in Urine

		percentage excretion[a] (w/w % of total)			
group	compd	0–4.5 h	4.5–25 h	25–49 h	0–49 h
CT (0.3 g/kg)	CT	1.217 ± 0.103	1.976 ± 0.366	0.057 ± 0.023	3.250 ± 0.333
	EC	ND[b]	ND	ND	ND
	3′-OMCT	0.378 ± 0.012	1.255 ± 0.261	0.022 ± 0.012	1.655 ± 0.256
	3′-OMEC	ND	ND	ND	ND
EC (0.3 g/kg)	CT	ND	ND	ND	ND
	EC	1.879 ± 0.312	0.798 ± 0.029	0.066 ± 0.046	2.743 ± 0.340
	3′-OMCT	ND	ND	ND	ND
	3′-OMEC	0.539 ± 0.041	0.447 ± 0.034	0.017 ± 0.009	1.002 ± 0.043
GSP (0.5 g/kg)	CT	0.040 ± 0.012	0.047 ± 0.028	0.003 ± 0.001	0.090 ± 0.003
	EC	0.034 ± 0.010	0.028 ± 0.001	0.002 ± 0.002	0.064 ± 0.004
	3′-OMCT	0.036 ± 0.010	0.065 ± 0.015	0.002 ± 0.001	0.102 ± 0.009
	3′-OMEC	0.009 ± 0.003	0.014 ± 0.004	0.002 ± 0.000	0.025 ± 0.005
GSPH (0.5 g/kg)	CT	0.002 ± 0.001	ND	ND	0.002 ± 0.001
	EC	0.003 ± 0.001	0.003 ± 0.001	0.001 ± 0.000	0.006 ± 0.000
	3′-OMCT	0.004 ± 0.001	ND	ND	0.004 ± 0.001
	3′-OMEC	0.002 ± 0.000	0.002 ± 0.001	0.001 ± 0.0002	0.005 ± 0.001

[a] Data are means ± SEM for four rats. [b] ND, not detected.

Table 6. Three Way ANOVA Analysis (Completely Randomized Design) of Urinary Excretion of the Monomer (CT, EC), GSP, and GSPH[a]

factor	sum of square	degree of freedom	mean of variance	Fcal	probability
A	28.8674	2	14.4337	73.3678	<0.00001
B	1.0862	2	0.5431	2.7607	0.06772
C	3.5235	3	1.1745	5.9702	0.00084
AB	4.3343	4	1.0836	5.5079	0.00045
AC	4.1293	6	0.6882	3.4983	0.00336
BC	6.5779	6	1.0963	5.5727	0.00005
ABC	10.6088	12	0.8841	4.4938	0.00001
error	21.2469	108	0.1967		
total	80.3744	143			

[a] A: collecting period (A1, 0–4.5 h; A2, 4.5–25 h; and A3, 25–49 h). B: Administered substance (B1, monomer (CT + EC); B2, GSP; and B3, GSPH). C: The excretion rate (w/w %) of each metabolite divided by the net amount of the corresponding monomer in each administered substance (C1, CT; C2, 3′-OMCT; C3, EC; and C4, 3′-OMEC).

Notably, in this study, we have elucidated the time course of the urinary excretion of GSP metabolites for the first time. More than 80% of the GSP metabolites detected was excreted within 25 h of GSP administration (**Figure 5**). GSP was excreted in urine as CT (0.055%), EC (0.040%), 3′-OMCT (0.088%), and 3′-OMEC (0.071%), which together accounted for only 0.254% of the administered dose, within 49 h of oral administration. These observations suggest that the bioavailability of GSP is low.

A method for the analysis of fecal GSP has not been established. The vanillin−HCl assay (*34*) cannot be used to determine fecal GSP because of colorization of the sample solutions derived from feces. The application of tannase (*37*) to the analysis of fecal GSP did not result in a good recovery of GSP (32.5%, **Table 2**), although the recovery of tannic acid from feces after tannase treatment was more than 80%. This variation in recovery might be due to differences in the chemical structure of tannic acid and GSP. Tannic acid is easily hydrolyzed by tannase to yield GA; by contrast, GSP is a condensed tannin that is not easily hydrolyzed. Some parts of galloylated GSP might be esters of GA and proanthocyanidins, which are easily hydrolyzed to yield GA. Although this analytical method was not efficient, 20.9% of the administered dose of GSP was detected in feces within 25 h, and 22.5% was detected within 49 h. These observations suggest that the majority of GSP might not be digested and might be excreted unaltered into feces.

From the above results, it is difficult to determine whether only the monomer constituents of GSP are absorbed and metabolized. We therefore administered GSP monomers (CT, EC, and GA), normal GSP, and GSPH orally to the rats and determined the serum concentration and urinary excretion of the metabolites. At 2.25 h after oral administration, it was considered likely that the serum metabolite concentrations would be relatively high, because maximal concentrations of serum metabolites were reached 3 h after the oral administration of GSP 1.0 g/kg (**Figure 4**). However, no metabolites were detected in the serum of the rats administered GSPH, although the corresponding metabolites were detected in the serum of the rats given CT, EC, GA, and GSP (**Figure 6**). For example, serum CT and 3′-OMCT were detected in rats given CT; serum EC and 3′-OMEC were detected in rats given EC; serum GA and 4-OMGA were detected in rats given GA; and serum CT, EC, 3′-OMCT, and 3′-OMEC were detected in rats given GSP (**Figure 6**). Furthermore, the serum concentrations of the last four metabolites increased with the dose of GSP (**Table 4**). Only trace amounts of CT, EC, 3′-OMCT, and 3′-OMEC were detected in urine of rats given GSPH, as compared with in the urine of rats given CT, EC, or GSP (**Figure 7**). Urinary excretion of the metabolites after the administration of CT or EC occurred relatively quickly (**Figure 7** and **Table 5**) and was similar to the urinary excretion observed after the administration of GSP at a dose of 1.0 g/kg (**Figure 5** and **Table 3**).

The bioavailability of the components of GSP was not high. Within 49 h of oral administration, CT was excreted in urine as CT (3.25%) and 3′-OMCT (1.66%), accounting for 4.91% of the administered dose; EC was excreted in urine as EC (2.74%) and 3′-OMEC (1.00%), accounting for 3.74%; GSP was excreted in urine as CT (0.090%), EC (0.064%), 3′-OMCT (0.102%), and 3′-OMEC (0.025%), accounting for 0.281%; and GSPH was excreted in urine as CT (0.002%), EC (0.006%), 3′-OMCT (0.004%), and 3′-OMEC (0.005%), accounting for 0.017%. To investigate the contribution of the monomer contained in each of the administered substances to the urinary excretion of metabolites, we divided the percentage excretion (w/w %, **Table 5**) of each metabolite by the net amount of the corresponding monomer in the administered substance and analyzed the results by three way ANOVA (completely randomized design). Of the three factors analyzed, corresponding

to the collecting period of urine, the administered substance, and the excretion rate (w/w %) of each metabolite divided by the net amount of the correspondent monomer in each administered substance, there was no statistical difference in the administered substance B (**Table 6**). This result strongly suggests that the urinary GSP metabolites are derived from the monomer components (CT and EC) of GSP and not from the decomposition and metabolism of the higher molecular components (proanthocyanidins).

Several researchers have investigated the metabolism of proanthocyanidins larger than dimers. Déprez et al. (*44*) reported that polymeric proanthocyanidins are catabolized into phenolic acids of low molecular weight by human colonic microflora. Spencer et al. (*45*−*47*) reported that polymeric proanthocyanidins are hydrolyzed by gastric fluid to lower molecular weight compounds such as CT and EC. By contrast, Donovan et al. (*32*) and Rios et al. (*48*) concluded that proanthocyanidins are not hydrolyzed by gastric acid. Rios et al. (*42*) suggested that these inconsistencies might be caused by differences in the experimental methods. They explained that the pH of gastric milieu in vivo does not decrease in the same way in vitro because of the gastric contents. Our results (**Table 6**) support the theory of Donovan et al. (*32*) that the high molecular weight proanthocyanidins in GSP are not bioavailable.

Therefore, it seems unlikely that the higher molecular components of GSP are decomposed to monomers such as CT and EC and then metabolized further. It seems more reasonable to suppose that only the monomeric components of GSP are absorbed and further metabolized and that these monomeric metabolites are thus responsible for the beneficial physiological properties of GSP observed in rats. However, it remains possible that some oligomers in GSP are absorbed from the intestinal tract and metabolized to monomers in the liver. In addition, the possibility that the unaltered or undigested higher molecular components of GSP have some beneficial physiological properties, for example, by proving dietary fiber, should not be ruled out.

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