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Effect on both aglycone and sugar moiety towards Phase II metabolism of anthocyanins

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Abstract

The effect of sugar moiety on anthocyanin metabolism was studied using anthocyanidin 3-rutinosides (cyanidin 3-*O*-rutinoside (Cy3R) and delphinidin 3-*O*-rutinoside (Dp3R)) and 3-*O*-glucosides (delphinidin 3-*O*-glucoside (Dp3G)). *O*-methylated Cy3R and Dp3R were detected in rat blood plasma after oral administration of Cy3R and Dp3R (100 mg/kg body weight). On the basis of HPLC retention time and UV–visible spectra together with the data of our previous studies on the hydrophobic metabolites of anthocyanidin 3-*O*-glucosides, it was concluded that both 3′- and 4′-*O*-methyl Cy3R were metabolites of Cy3R. On the other hand, only 4′-*O*-methyl Dp3R was detected as hydrophobic metabolite of Dp3R. A group of hydrophilic metabolites was also detected in rat blood plasma after oral administration of anthocyanins (Dp3G, Cy3R and Dp3R) and their structures were determined to be extended glucuronides and their *O*-methyl analogues by tandem MS analysis. The amounts of extended glucuronides of Dp3G, Cy3R and Dp3R were less than those of cyanidin 3-*O*-glucoside (Cy3G) reported in our previous study. On the other hand, anthocyanidin–glucuronides (both cyanidin–glucuronide and delphinidin–glucuronide) were not detected after oral administration of Cy3R, Dp3R and Dp3G. These results indicated that both the type of sugar moiety and stability of aglycone largely affected phase II metabolism of anthocyanins, and also indicated that the type of sugar moiety did not affect the *O*-methylation metabolism but affected glucuronyl conjugation in both liver and small intestine.
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Keywords: Anthocyanidin 3-*O*-rutinoside; Anthocyanidin 3-*O*-glucoside; Cyanidin; Delphinidin; Rutinoside–glucuronide; Glucoside–glucuronide; Extended glucuronide; Metabolism

1. Introduction

Anthocyanins are coloured compounds widely distributed in edible plants including berries (Ichiyanagi, Hatano, Matsugo, & Konishi, 2004a; Matsumoto, Hanamura, Kawakami, Sato, & Hirayama, 2001a; Garcia-Viguera, Zafrilla, & Tomas-Barveran, 1998), crops (Ichikawa et al., 2001; Noda, Kaneyuki, Igarashi, Mori, & Packer, 2000) and vegetables (Ichiyanagi, Terahara, Rahman, & Konishi, 2006a; Kammerer, Carle, & Schieber, 2004). Recently, numerous studies on functionality of anthocyanins have been reported such as vision improvement (Mercier, Perdriel, Rozier, & Cheraleraud, 1965; Matsumoto, Nakamura, Tachibanaki, Kawamura, & Hirayama, 2003), antioxidant activity (Rahman, Ichiyanagi, Komiyama, Hatano, & Konishi, 2006; Ichiyanagi, Hatano, Matsugo, & Konishi, 2003; Ichiyanagi, Hatano, Matsugo, & Konishi, 2004b; Ichiyanagi, Hatano, Matsugo, & Konishi, 2004c; Kahkonen & Heinonen, 2003) and anti-cancer property (Chen et al., 2006; Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003), thus, anthocyanins have

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attracted much attention as functional food factors. However, to understand health beneficial effects of anthocyanins, studies on their absorption and metabolism are critical. Several studies indicated that anthocyanins were absorbed and detected as their intact glycoside forms in blood plasma of human (Wu, Cao, & Prior, 2002; Felgines et al., 2003; Mazza, Kay, Cottrell, & Holub, 2002; Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001) or experimental animals (Felgines et al., 2007; Suda et al., 2002; Morazzoni, Livio, Scilingo, & Malandrino, 1991; Matsumoto et al., 2001a). Metabolism of anthocyanin has also been reported by several groups (Felgines et al., 2003; Wu, Pittman, & Prior, 2004). We have previously reported that delphinidin 3-*O*-glucoside (Dp3G), a potent antioxidant in bilberry, was metabolized to 4′-*O*-methyl Dp3G (Ichiyanagi et al., 2004d), whereas, cyanidin 3-*O*-glucoside (Cy3G) produced both 3′ and 4′-*O*-methyl Cy3G (Ichiyanagi et al., 2005a). This indicates that the structure of anthocyanin will modulate functionality of anthocyanins in vivo.

On the other hand, it is widely accepted that glucuronyl conjugation, typical phase II metabolism, is another major metabolic path of flavonoids (Gee et al., 2000; Hollman, de Vries, van Leeuwen, Mengelers, & Katan, 1995). This indicates that intestinal flora plays an important role in metabolism when foods containing flavonoids are orally ingested. In fact, cyanidin–glucuronide produced by intestinal enzymes has been identified as one of the metabolites of Cy3G in our previous study (Ichiyanagi et al., 2005a), although plasma glucuronide level was quite low compared with that of other flavonoids such as epicatechin (Natsume et al., 2003) and quercetin (Hollman et al., 1995). We have also reported the novel metabolic fate of anthocyanin, extended glucuronidation (glucoside–glucoronide) which is specific phase II metabolic route for anthocyanins absorbed as their intact glycoside forms (Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2005b). Extended glucuronidation mainly occurred in liver not in small intestine and plasma level of extended glucuronides as a whole was about 40% of metabolites (Ichiyanagi et al., 2005b). This fact indicated that phase II metabolism is another important biological modification for anthocyanins. However, studies on relationship between anthocyanin structure and phase II metabolism (glucuronidation and extended glucuronidation) have not been reported. Thus, in the present study, phase II metabolism of three types of anthocyanins were precisely examined together with *O*-methylation reaction of anthocyanins and the results were compared with those of Cy3G reported previously (Ichiyanagi et al., 2005b) to clarify the effect of aglycone structure and sugar moiety on anthocyanin metabolism in rats.

2. Materials and methods

2.1. Chemicals

Anthocyanins (Dp3G, cyanidin 3-*O*-rutinoside (Cy3R) and delphinidin 3-*O*-rutinoside (Dp3R), Fig. 1) were purified from blackcurrant (*Ribes nigrum* L.) extract according to a previously described method (Matsumoto et al., 2001a). All other reagents including trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan) and used without any further purification. An authentic sample of peonidin 3-*O*-glucoside (Pn3G) was purified from a bilberry species (*Vaccinium myrtillus* L.) according to a previously described method with a slight modification (Ichiyanagi et al., 2004e). Briefly, bilberry extract was separated by open column packed with MCI gel (4.5 cm × 45 cm) (Mitsubishi Chemical Co. Ltd., Tokyo, Japan) with aqueous methanol solution (methanol contents increased from 0 to 100%). Pn3G containing fraction thus obtained was further purified by semi preparative HPLC with Develosil ODS HG-5 column (20 mm × 250 mm, Nomura Chemical Co. Ltd., Aichi, Japan) using aqueous solution containing 0.5% TFA and 13% acetonitrile as elution solvent. The peak fraction was evaporated to dryness in vacuo and stored at −80 °C until use.

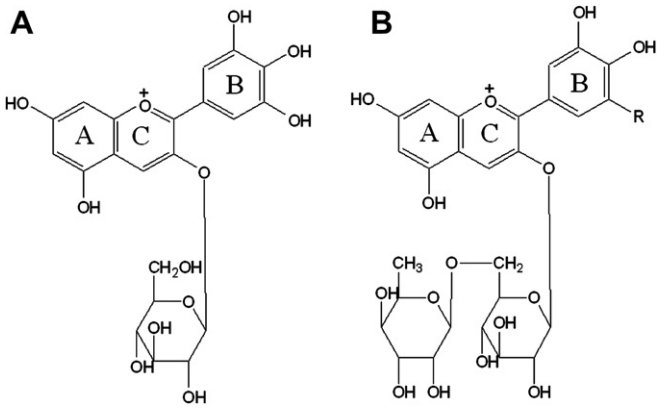

Fig. 1. Chemical structures of Dp3G, Cy3R and Dp3R. (A) Dp3G and (B) Cy3R and Dp3R R = H: cyanidin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoisde (cyanidin 3-*O*-rutinoside) R = OH: delphinidin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoisde (delphinidin 3-*O*-rutinoside).

2.2. Animals and diets

SPF male Wistar ST rats (5 weeks old, 160 g body weight) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and individually housed in stainless-steel wire-mesh cages at 23 ± 1°C for conditioning under a 12-h light/dark cycle. The rats were allowed free access to tap water and a control diet for 7 days before the experiment. The rats were treated in accordance with the Guidelines of Niigata University of Pharmacy and Applied Life Sciences.

2.3. Experimental design and plasma preparation

After 7 days adaptation, twelve rats were cannulated with a polyethylene tube (PE-50) into a neck vein under anesthesia with diethyl ether according to our previously

described method (Ichiyanagi et al., 2005a; Ichiyanagi et al., 2005b). Briefly, neck vein was isolated and a small hole was made by the use of scissors to insert a polyethylene tube (PE-50). After insertion of the tube in the hole, the vein and tube were occluded and inserted tube was penetrated through the skin and guided out from the back of the rats. After 24 h starvation, the rats were randomly assigned into four groups, and each anthocyanin (Dp3G, Cy3R or Dp3R) dissolved in aqueous 0.1% citric acid solution was orally administrated (100 mg/kg body weight). During the experiment, the rats were allowed to move freely in the wire-mesh floor cages. Blood sample was collected before oral administration and 15, 30, 60, 120, 240 and 480 min after administration from the cannulated tube using heparinized syringe. Donor blood was obtained from the inferior vena cava of other healthy rats by use of needle and syringe containing sodium citrate (500 μL of aqueous 10% sodium citrate solution for 8 mL blood) under anesthesia with diethyl ether. After withdrawal of the blood sample (600 μL), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000*g* for 5 min at 4 °C for HPLC analysis.

2.4. Determination of anthocyanins and their metabolites in the plasma

Anthocyanin and its metabolites were extracted from plasma samples by use of a Sep-Pak C_{18} cartridge light column (Waters, Manchester, UK), essentially according to the method we previously reported (Ichiyanagi et al., 2005a, 2005b). Briefly, the plasma samples (300 μL) were applied to Sep-Pak C_{18} cartridges conditioned with methanol (2 mL) and aqueous solution containing 3% TFA (2 mL). After the sample application, the cartridges were washed successively with 2 mL of aqueous solution containing 3% TFA, dichloromethane, benzene, and then anthocyanins and their metabolites were eluted with aqueous solution containing 1% TFA and 50% acetonitrile (1 mL). The effluents were evaporated to dryness *in vacuo* and dissolved in 150 μL of aqueous solution containing 0.5% TFA. The sample solution was passed through Centricut (0.45 μm, Kurabou Industries Ltd., Osaka, Japan) and then analyzed by HPLC according to our previous report (Ichiyanagi et al., 2005b). Briefly, aliquots (50 μL) of the sample solutions were injected into the HPLC system (Hitachi 7200) equipped with Develosil ODS HG-5 column (Nomura Chemical Co. Ltd., Aichi, Japan, 1.0 mm × 150 mm) using aqueous solution containing 0.5% TFA and 18% methanol as elution solvent for the analysis of anthocyanins and *O*-methyl metabolites. The recovery of anthocyanins and their metabolites in this method was checked using authentic Dp3G, Cy3R and Dp3R and they were 83.2, 83.5 and 83.2%, respectively. For the analysis of extended glucuronides, aqueous solution containing 0.5% TFA and 5% methanol was used as the elution solvent at a flow rate of 0.1 mL/min, and the elution peaks were monitored at 520 nm with a UV–visible detector (Hitachi Co. Ltd., Tokyo, Japan).

2.5. Identification of anthocyanins and their metabolites

Anthocyanin metabolites were isolated by the similar HPLC method described above using Develosil ODS HG-5 column (Nomura Chemical Co. Ltd., Aichi, Japan, 4.6 mm × 150 mm) and the metabolites were identified by TOF MS–MS spectrometry using Q-Tof Ultima (Waters, Manchester, UK). The conditions for TOF MS-MS are as follows: A syringe pump (Single Syringe Pump, KD Science Inc., Massachusetts, USA) was used to provide a constant infusion (300 μL/h) of the sample into the MS ion-source. MS parameters used were as follows: 3.2 kV for capillary; 9.1 kV for reflection. Argon gas was used for collision at pressure of 11 psi and applied voltage was 24 kV.

3. Results

Fig. 2 shows typical HPLC chromatograms of rat blood plasma 30 min after oral administration of anthocyanins (A for Dp3G, B for Cy3R and C for Dp3R, respectively). Although the data are not shown, each anthocyanin showed similar maximum plasma level (0.29 ± 0.06 μM for Dp3G, 0.21 ±;0.03 μM for Cy3R and 0.27 ± 0.03 μM for Dp3R, respectively: values are means ± SEM of four rats). The maximum plasma concentration after oral administration of anthocyanin was attained at 15 min for Dp3G and 30 min for both Cy3R and Dp3R, respectively. In Fig. 2, several new peaks presumably due to hydrophobic metabolites of each anthocyanin were detected which eluted slower than intact anthocyanins (peak 2, 4, 5 and 7) and groups of hydrophilic metabolites were also detected (marked in circle). The HPLC chromatograms of hydrophilic metabolites are also shown in Fig. 3 (A for Dp3G, b for Cy3R and C for Dp3R, respectively).

In order to obtain structural information about the metabolites, each peak was collected by HPLC and successively analyzed by tandem MS. The mass values of each metabolite and their fragments thus obtained are summarized in Table 1. MS fragments of peak 2 well agreed with those of *O*-methyl Dp3G (*m*/*z* 479) and its aglycone, *O*-methyl delphinidin (*m*/*z* 317). MS fragments of both peak 4 and 5 were 609 for molecular ion, 463 and 301 for fragment peaks. These values are in good agreement with those of *O*-methyl Cy3R, *O*-methyl Cy3G and aglycone (*O*-methyl cyanidin), respectively. Similarly, MS fragments of peak 7 agree with those of *O*-methyl Dp3R (625 for molecular ion, 479 for mono-*O*-methyl Dp3G and 317 for aglycon, *O*-methyl delphinidin). To clarify methylation site of metabolites, UV–visible spectra of peak 4, 5 and 7 were measured together with those of authentic Pn3G and Dp3R and the results are shown in Fig. 4 and **5**. As shown in Fig. 4, maximum absorption wavelength of Dp3R appeared at 520 nm. Maximum absorption wave-

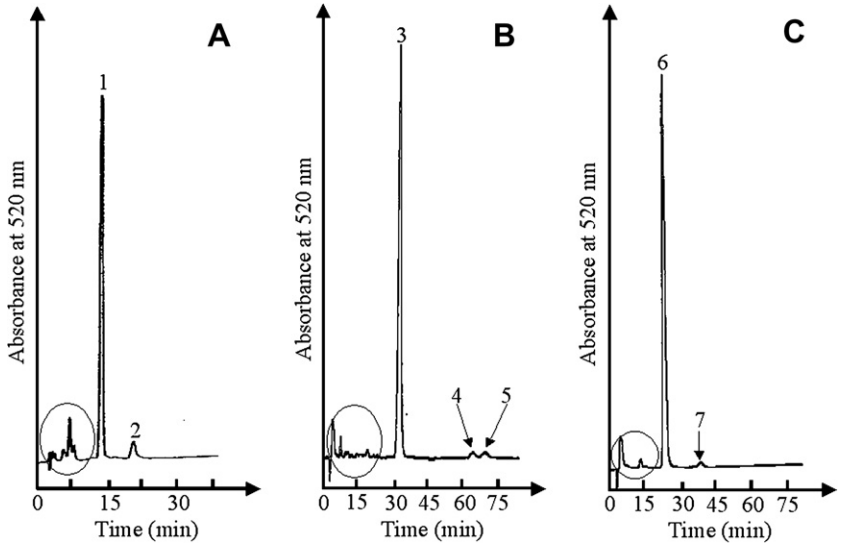

Fig. 2. Typical HPLC chromatogram of rat blood plasma 30 min after oral administration of anthocyanins. (A) Dp3G; (B) Cy3R and (C) Dp3R. Hydrophilic metabolites of each anthocyanin are marked in circle.

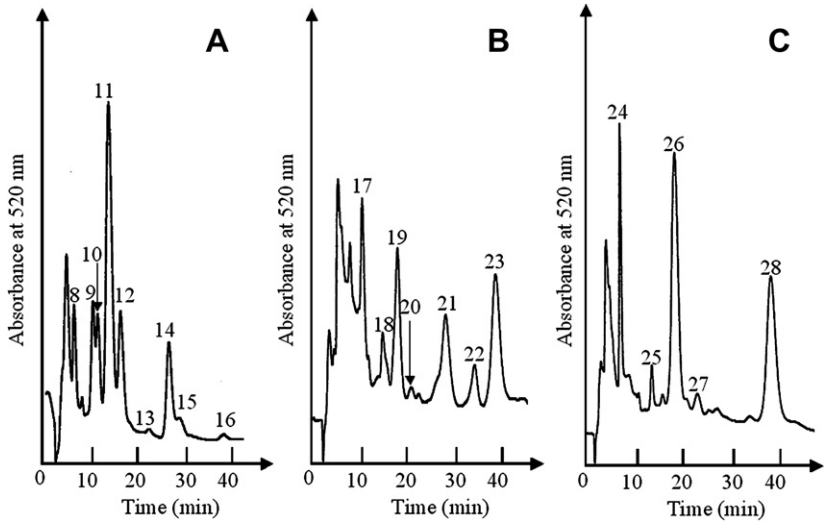

Fig. 3. Typical HPLC chromatogram of hydrophilic metabolites in rat blood plasma 15 min after oral administration of anthocyanins. (A) Dp3G; (B) Cy3R and (C) Dp3R.

length at 499 nm of peak 7 strongly indicates that methylation occurred at phenolic OH group at 4′ position of the delphinidin B ring, since the same spectral shift was observed for 4′-*O*-methyl Dp3G in our previous study (Ichiyanagi et al., 2004c). Therefore, metabolite of peak 7 was identified to be 4′-*O*-methyl Dp3R. On the other hand, only a small spectral shift was observed for Cy3R metabolites (maximum wavelength of Cy3R, peak 4 and 5 were 513, 510 and 513 nm, respectively, Fig. 5). The spectrum of peak 5 was identical with that of authentic Pn3G which indicated that peak 5 carries peonidin as aglycone. Therefore, methylation site of peak 5 was assigned to phenolic OH group at 3′ position of aglycone (cyanidin). Although the difference was fairly small, shorter wavelength shift was also observed for the maximum wavelength of peak 4 indicating that *O*-methylation occurred at 4′ position on hydroxyl group.

The MS fragments of hydrophilic metabolites of anthocyanins were also obtained and the results are shown in Table 1. The MS fragments of hydrophilic metabolites were 641/465/303 for peak 9, 12 and 14, 655/479/317 for peak 10, 11 and 13, 771/595/449/287 for peak 20 and 22, 785/609/463/301 for peak 19, 21 and 23, 787/611/465/303 for peak 26 and 801/625/479/317 for peak 25 and 28, respectively. These values were agreed with those of extended glucuronides of Dp3G, Cy3R, Dp3R and *O*-methyl analogues of extended glucuronides.

4. Discussion

Anthocyanins are one of the classes of flavonoids, which show variety of functionalities (Chen et al., 2006; Ichiyanagi et al., 2003, 2004b, 2004c; Kahkonen & Heinonen, 2003; Matsumoto et al., 2003; Mercier et al., 1965; Rahman

Table 1
Peak identification of anthocyanin metabolites

Peak number	Type of metabolite	MS–MS fragment
1	Delphinidin 3-*O*-D-glucopyranoside	465/303
2	4′-*O*-Methyl delphinidin 3-*O*-D-glucopyranoside	479/317
3	Cyanidin 3-*O*-rutinoside	595/449/287
4	4′-*O*-Methyl cyanidin 3-*O*-rutinoside	609/463/301
5	3′-*O*-Methyl cyanidin 3-*O*-rutinoside	609/463/301
6	Delphinidin 3-*O*-rutinoside	611/465/303
7	4′-*O*-Methyl delphinidin 3-*O*-rutinoside	625/479/317
8	Unknown	–
9	Delphinidin 3-*O*-D-glucopyranoside–glucuronide	641/465/303
10	4′-*O*-Methyl delphinidin 3-*O*-D-glucopyranoside–glucuronide	655/479/317
11	4′-*O*-Methyl delphinidin 3-*O*-D-glucopyranoside–glucuronide	655/479/317
12	Delphinidin 3-*O*-D-glucopyranoside–glucuronide	641/465/303
13	4′-*O*-Methyl delphinidin 3-*O*-D-glucopyranoside–glucuronide	655/479/317
14	Delphinidin 3-*O*-D-glucopyranoside–glucuronide	641/465/303
15	Unknown	–
16	Unknown	–
17	Unknown	–
18	Unknown	–
19	*O*-Methyl cyanidin 3-*O*-rutinoside–glucuronide	785/609/463/301
20	cyanidin 3-*O*-rutinoside–glucuronide	771/595/449/287
21	*O*-Methyl cyanidin 3-*O*-rutinoside–glucuronide	785/609/463/301
22	cyanidin 3-*O*-rutinoside–glucuronide	771/595/449/287
23	*O*-Methyl cyanidin 3-*O*-rutinoside–glucuronide	785/609/463/301
24	Unknown	–
25	4′-*O*-Methyl delphinidin 3-*O*-rutinoside–glucuronide	801/625/479/317
26	Delphinidin 3-*O*-rutinoside–glucuronide	787/611/465/303
27	Unknown	–
28	4′-*O*-Methyl delphinidin 3-*O*-rutinoside–glucuronide	801/625/479/317

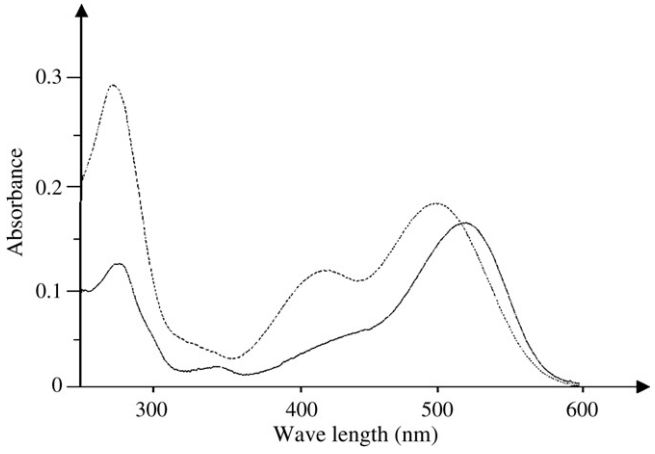

Fig. 4. UV–visible spectrum of Dp3R and its metabolite. — Dp3R, ··· MDp3R.

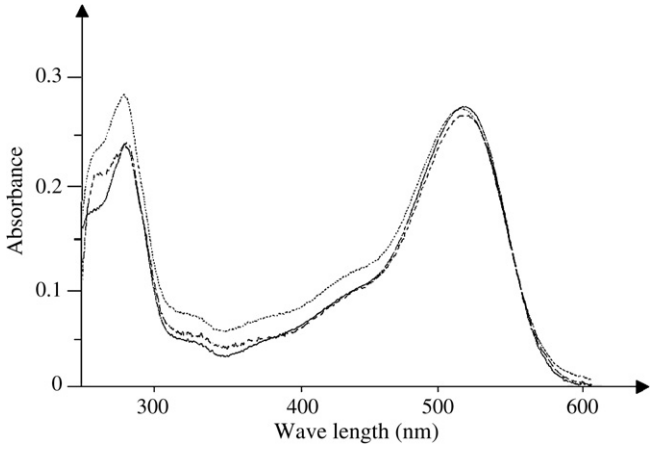

Fig. 5. UV–visible spectrum of Cy3R and its metabolites. ··· MCy3R-1, — MCy3R-2, --- Pn3G.

et al., 2006). In order to evaluate health beneficial effect of anthocyanins, it is important to study gastrointestinal uptake and their metabolism. Thus, numerous studies on absorption of anthocyanins have been reported in last decade (Cao et al., 2001; Felgines et al., 2003, 2007; Matsumoto et al., 2001b; Mazza et al., 2002; Morazzoni et al., 1991; Suda et al., 2002; Wu et al., 2004). The anthocyanin metabolism has also studied in past several years (Felgines et al., 2003; Felgines et al., 2007; Wu et al., 2004) and it is widely accepted that *O*-methylation is major metabolism for flavonoids including anthocyanins (Natsume et al., 2003; Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999). Miyazawa et al. (1999) reported that two *O*-methyl Cy3G were the major metabolites in rat liver and kidney after oral administration of Cy3G rich fruit extract, although the methylation site was not determined. We have reported that metabolism of Dp3G in rats and metabolite was determined to be 4′-*O*-methyl Dp3G (Ichiyanagi et al., 2004d), on the other hand, Cy3G was metabolized to both 4′ and 3′-*O*-methyl Cy3G in rats (Ichiyanagi

et al., 2005a). Therefore, it was concluded that the number of phenolic OH group on aglycone B ring determined *O*-methylation site of anthocyanidin 3-*O*-glucoside.

Another major metabolic path of flavonoids is glucuronidation (Hollman et al., 1995; Natsume et al., 2003). Normally, flavonoids such as epicatechin and quercetin undergo this metabolism. We have reported presence of cyanidin–glucuronide (4.4% in urinary excreted anthocyanins) both in urine and blood plasma, although its quantity was quite small compared with those of other flavonoids (Ichiyanagi et al., 2005a). Felgines et al. (2003) also reported that glucuronyl conjugates were the major metabolites of pelargonidin 3-*O*-*β*-D-glucpyranoside (Pg3G) in human excreted urine after ingestion of strawberry fruits, thus it is deduced that aglycone structure is the modulator for glucuronidation of anthocyanidins.

Recently, we have reported production of hydrophilic metabolites of Cy3G in both rat urine and blood plasma after single oral administration of purified Cy3G and they were finally assigned to extended glucuronides of Cy3G and its *O*-methyl analogues (Ichiyanagi et al., 2005b). We also confirmed that extended glucuronides were mainly produced in liver by phase II enzymatic reaction after uptake of Cy3G in blood plasma and the sum of these metabolites was about 40% of urinary excreted anthocyanins. Although metabolism of anthocyanidin 3-*O*-glucoside was precisely studied in past few years, studies on biotransformation of anthocyanidin 3-*O*-rutinosides are limited (Matsumoto et al., 2006). A large number of studies on absorption and metabolism of quercetin glycosides (quercetin 3-glucoside, quercetin 3-rahmnoside and rutin (quercetin 3-rutinoside)) have been reported (Gee et al., 2000; Hollman et al., 1995; Natsume et al., 2003) and it was concluded that the type of sugar moiety largely affected metabolism of quercetin glycosides. We have suggested t hat the type of sugar moiety may modulate biological behavior of anthocyanins in our previous study (Ichiyanagi, Shida, Rahman, Hatano, & Konishi, 2006b). Thus, the aim of this study was set to clarify the effects of sugar moiety on anthocyanin metabolism in rats.

Matsumoto et al. (2006) reported high production of 4′-*O*-methy Dp3R in excreted rat bile after single oral injection of purified Dp3R. The production of *O*-methyl Cy3R has also been reported by Wu et al. (2004) although the methylation site was not discussed. In the present study, two hydrophobic metabolites of Cy3R and one hydrophobic metabolite of Dp3R were detected in rat blood plasma after oral administration of each anthocyanin, they eluted slower than intact anthocyanins. The molecular MS peak and MS fragments of these hydrophobic metabolites well agreed with those of *O*-methyl metabolites of Cy3R (609/463/301 for peak 4 and 5) and Dp3R (625/479/317 for peak 7). The maximum wavelength of UV–visible absorption spectrum of peak 5 was identical to that of authentic Pn3G, thus it was concluded that peak 5 carries peonidin (3′-*O*-methyl cyanidin) as aglycone. On the other hand, the maximum wavelength of peak 7 largely shifted to shorter wavelength indicating *O*-methylation occurred at 4′ hydroxyl group on delphinidin similar to the case of our previous result on Dp3G (Ichiyanagi et al., 2004d). Although the difference was fairly small, shorter wavelength shift was also observed for the maximum wavelength of peak 4 indicating that *O*-methylation occurred at 4′ position on hydroxyl group. Significantly shorter HPLC retention time of peak 4 than that of peak 5 also supported this idea. Thus, it was concluded that the type of sugar moiety did not affect the *O*-methylation site of anthocyanins.

Recently, production of Cy3G has been reported when Cy3R was incubated with human gut microflora (Aura et al., 2005). The report concluded that Cy3R was hydrolyzed and released Cy3G by α-rhamnosidase produced by intestinal bacteria. Released Cy3G was further metabolized to Cy by *β*-glucosidase. However, in the present study, Cy3G was not observed in rat blood plasma as metabolite of Cy3R after oral administration of purified Cy3R. Also, Dp3G was not detected as the metabolite of Dp3R. Presumably, only a limited part of anthocyanidin 3-*O*-rutinosides were hydrolyzed by α-rhamnosidase in small intestine, consequently even a trace of anthocyanidin 3-*O*-glucosides could not be detected in blood plasma as metabolites of anthocyanidin 3-*O*-rutinosides.

In the present study, groups of hydrophilic metabolites were also detected after oral administration of Cy3R, Dp3R and Dp3G (Fig. 2), similar to our previous results on study of Cy3G. From the MS fragments of isolated peaks, it was clarified that these metabolites were extended glucuronides of Cy3R, Dp3R, Dp3G and their *O*-methyl analogues (Table 1). However, these plasma levels were largely different from one another depending on the types of anthocyanins. The number and amounts of extended glucuronides of Dp3G were several times smaller than those of Cy3G. Moreover, amounts of extended glucuronides of Cy3R were 1/10 smaller than those of Cy3G, and Dp3R showed a similar tendency. Therefore, it was concluded that both types of sugar moiety and B ring structure modulated extended glucuronidation of anthocyanins.

We previously reported (Ichiyanagi et al., 2005a) the production of Cy-glucuronide as a metabolite of Cy3G in rat blood plasma and urine after oral administration of Cy3G. In the present study, glucuronidation of anthocyanidins has also been evaluated. However, no cyanidin- or delphinidin–glucuronide was detected in rat blood plasma after oral administration of Cy3R or Dp3R, and this result indicated that sugar moiety modulated the glucuronidation of anthocyanidin. Production of delphinidin–glucuronide (Dp-glucuronide) from Dp3G was also surveyed, however, no Dp-glucuronide was observed in rat blood plasma as a metabolite of Dp3G. Perhaps, Dp produced from Dp3G was quite unstable under intestinal environments, thus, substantially all Dp should be degraded into ring fission products, although the structures of degradation products are still unclear. Thus, it was presumed not only the type of sugar moiety but also aglycone

structure, more correctly, aglycone stability affected the glucuronidation reaction of anthocyanidins.

Fig. 6 showed comparison of anthocyanin metabolism expressed as area under the plasma concentration curve (AUC) ratio of each metabolite. As shown in Fig. 6, both sugar moiety and aglycone structure did not show significant difference ($P < 0.05$) in *O*-methylation of anthocyanins. Cy-glucuronide was detected as metabolite only in the case Cy3G and could not be detected as metabolite of Cy3R. Therefore, sugar moiety is the modulator for glucuronidation of anthocyanidin. However, Dp-glucuronide could not be detected in both case of Dp3G and Dp3R, thus, aglycone structure also modulated glucuronidation of anthocyanidins. On the other hand, all anthocyanins tested in the present study metabolized to extended glucuronides, although their amounts were largely different from one another depending on both aglycone and sugar moiety of anthocyanins. Especially, this metabolic route was minor for anthocyanins carrying delphinidin as aglycone and anthocyanins bonded rutinose as sugar moiety.

The metabolic fate of anthocyanidin 3-*O*-rutinosides deduced as follows: first orally administered anthocyanins were absorbed from small intestine. However, release of aglycone due to hydrolysis in small intestine was thought to be a minor route for anthocyanidin 3-*O*-rutinosides. The absorbed Cy3R is then methylated by catechol-*O*-methyl transferase in liver, yielding 3′ and 4′-*O*-methyl Cy3R. Dp3R was also metabolized to 4′-*O*-methyl Dp3R in liver. Thus, the aglycone structure mainly determined the methylation site of anthocyanins. On the other hand, no glucuronyl conjugate was detected in both Cy3R and Dp3R administered rat plasma. Wu, Pittman, Mckay, and Prior (2005) observed Cy-glucuronide formed after oral administration of fruits extract containing Cy3G and Cy3R but could not conclude which was the major substrate for enzymatic reaction of UDP-glucuronyl transferase. In the present study, we clearly demonstrated that Cy-glucuronide was produced from Cy3G and not from Cy3R using authentic anthocyanins. Some parts the absorbed Cy3R and Dp3R metabolized to extended glucuronides in the liver but their amount was much low compared with those of Cy3G.

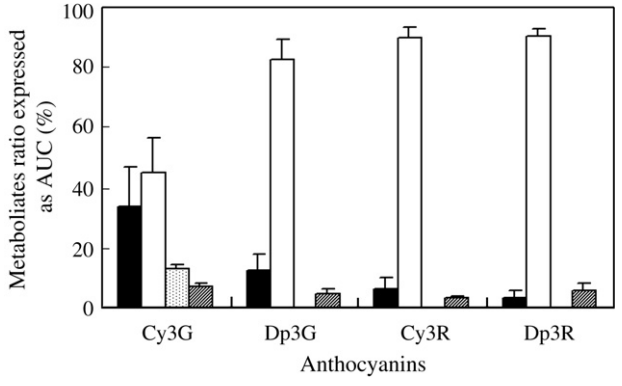

Fig. 6. Comparison of anthocyanin metabolism expressed as AUC ratio (%) in rat blood plasma. Symbols: ■ extended glucuronides, □ intact anthocyanin, □ anthocyanidin–glucuronide, ▨ *O*-methyl anthocyanins Values are means ± SD of four rats.

Thus, the type of sugar moiety did not affect on the *O*-methylation of anthocyanin B ring but modulated the step of glucuronyl conjugation. Both attached sugar moiety and stability of aglycone affected phase II metabolism of anthocyanins and therefore, modulated functionality of anthocyanins in vivo. Also, we recently reported (Ichiyanagi, Rahman, Hatano, Konishi, & Ikeshiro, 2007) protocatechuic acid is not a major metabolite of Cy3G in rat blood plasma after oral administration of purified Cy3G. This indicated degradation products of anthocyanins produced in biological systems are still unclear. Thus, further studies on degradation of anthocyanins in vivo are required to clarify the functionality of anthocyanins.

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