

Regular Article

Effect of Acylated Sugar Moieties on Gastrointestinal Absorption of Mono-Acylated Anthocyanins in Rats

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In this study, five types of mono-acylated anthocyanins were isolated from colored-fleshed potato (*Solanum tuberosum* L.) and black carrot (*Daucus carota* subsp. *sativus*), and their gastrointestinal absorption was evaluated based on the plasma profiles of rats. The absorption amounts of mono-acylated anthocyanins were slightly higher or at least similar to those of non-acylated anthocyanins, which carry the same aglycone, when the purified anthocyanins were orally administered. The absorption amounts of petunidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*- β -D-glucopyranoside isolated from purple-fleshed potato were 1.3 to 2.3-fold higher than those of three types of cyanidin 3-*O*-(2''-xylopyranosyl-6''-*O*-(6'''-*O*-acyl- β -D-glucopyranosyl))- β -D-galactopyranosides isolated from black carrot, whereas pelargonidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*- β -D-glucopyranoside isolated from red-fleshed potato exhibited an exceptionally poor absorption profile, as demonstrated by the comparison of the area under the plasma concentration curves during 8 h after oral administration normalized to the orally administered dose. The gastrointestinal absorption of mono-acylated anthocyanins was tended to determine by their structures, including aglycone moieties, attached sugars, and the balance of the entire molecule. Our findings suggested that the branched acylated sugars attached to position 3 tended to suppress the absorption of mono-acylated anthocyanins from the gastrointestinal tract. Furthermore, the poor intestinal absorption of mono-acylated anthocyanins in the mixtures was likely due to the competitive absorption of these molecules with contaminants that co-occur in plant materials such as organic acids.

Key words mono-acylated anthocyanin, absorption, branched-chain, aglycone, aromatic acyl moiety, whole-molecule balance

INTRODUCTION

Anthocyanins are reddish pigments classified within the flavonoid family that are distributed in various colored fruits and vegetables.¹⁾ Anthocyanins are commonly found in their glycoside or acylated glycoside forms in plants and six major anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin) are known as the aglycones of anthocyanins.¹⁾ Numerous *in vivo*²⁾ and *in vitro*³⁾ studies have suggested that anthocyanins may play an important role in human disease prevention as functional food components. Studies on the bioavailability of non-acylated anthocyanins such as absorption,^{4,5)} metabolism,^{6,7)} distribution,⁸⁾ and excretion⁹⁾ have also clarified the biological function of anthocyanins. These studies have demonstrated that the gastrointestinal absorption and metabolic fates of non-acylated anthocyanins are influenced by their structures, particularly their aglycone moieties.⁴⁻⁹⁾

Acylated anthocyanins with acyl moieties in their structures are the other group of anthocyanins found in colored vegetables such as eggplant,¹⁰⁾ purple-fleshed sweet potato,¹¹⁾ red

cabbage,¹²⁾ and black carrot,¹³⁾ as well as in the petals of various flowers.¹⁴⁾ Normally, acylated anthocyanins are further categorized into two groups depending on the aliphatic or aromatic acyl groups attached to their molecular structures.¹⁵⁾ Most acylated anthocyanins with aromatic acyl groups are relatively more stable than non-acylated anthocyanins due to the intramolecular hydrophobic stacking between anthocyanidin and the attached aromatic acyl moieties.¹⁵⁾ Particularly, poly-acylated anthocyanins are quite stable in neutral aqueous solutions¹⁶⁾ and are therefore used as safe food colorants. Additionally, recent studies have suggested that acylated anthocyanins could possess several health-promoting properties.^{15,17)}

Studies on the bioavailability of acylated anthocyanins have also been conducted to characterize the *in vivo* functions of acylated anthocyanins.¹⁸⁻²²⁾ We have previously reported that the absorption of nasunin (delphinidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*- β -D-glucopyranoside), a common acylated anthocyanin isolated from eggplant (*Solanum melongena*), was similar or even higher than that of delphinidin 3-*O*- β -D-glucopyranoside (Dp3G), a non-acylated anthocyanin carrying the same agly-

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cone, when the purified anthocyanins were orally administered to rats.¹⁸⁾ In contrast, low absorption of acylated anthocyanins has been reported using plant extract or juice as anthocyanin sources.^{19–22)} For example, peonidin 3-*O*-(2''-*O*-β-D-glucopyranosyl-6''-*O*-caffeoyl)-β-D-glucopyranosyl-5-*O*-β-D-glucopyranoside (Pn3GCafG5G) derived from purple-fleshed sweet potato (*Ipomoea batatas* L.) exhibited an exceptionally low absorption profile in both rat²¹⁾ and human studies²²⁾ after the administration of purple-fleshed sweet potato extracts. These results suggest that the difference in chemical structures of acylated anthocyanins such as the types of aglycone, attached acylated sugar types, and various combinations of these factors complicate the absorption of acylated anthocyanins by the gastrointestinal tract. Moreover, it was reported that certain components in food affect the gastrointestinal absorption of acylated anthocyanins.²³⁾ Therefore, studies using purified anthocyanins are necessary to clarify the relationship between structures of acylated anthocyanin and gastrointestinal absorption.

Varieties of colored-fleshed potato (*Solanum tuberosum* L.) are known as anthocyanin-containing plants. The structures of the major mono-acylated anthocyanins in the red-fleshed potato (Inca-red) and purple-fleshed potato (Inca-purple) were found to be pelanin (pelargonidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside) and petanin (petunidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside),²⁴⁾ respectively, which are the same analogs of nasunin (delphinidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside),¹⁰⁾ a typical mono-acylated anthocyanin (Fig. 1A). Moreover, black carrot (*Daucus carota subsp. sativus*), a native plant in central Asia, is also a popular anthocyanin-containing plant. Particularly, this plant contains three types of mono-acylated anthocyanins with cyanidin as an aglycone: cyanidin 3-*O*-(2''-xylopyranosyl-6''-*O*-(6'''-*O*-*p*-coumaroyl-β-D-glucopyranosyl))-β-D-galactopyranoside (Cy3XylpCoGGal), cyanidin 3-*O*-(2''-xylopyranosyl-6''-*O*-(6'''-*O*-feruloyl-β-D-glucopyranosyl))-β-D-galactopyranoside (Cy3XylFerGGal), and cyanidin 3-*O*-(2''-xylopyranosyl-6''-*O*-(6'''-*O*-sinapoyl-β-D-glucopyranosyl))-β-D-galactopyranoside (Cy3XylSinGGal)¹³⁾ (Fig. 1B). The contents of mono-acylated anthocyanins in colored-fleshed potato and black carrot are relatively high. Therefore, both plants are suitable for the isolation of mono-acylated anthocyanins on a large scale.

In this study, five types of the above-described mono-acylated anthocyanins were isolated from colored-fleshed potato and black carrot, after which their gastrointestinal absorption was evaluated in rats. Furthermore, the results were compared with those in previous reports of acylated and non-acylated anthocyanin absorption to discuss the effect of aglycone, aromatic acyl groups, and attached sugar moieties on anthocyanin absorption in the gastrointestinal tract.

MATERIALS AND METHODS

Reagents HPLC-grade acetonitrile was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). All other reagents, including trifluoroacetic acid (TFA), were purchased from Fuji Film Wako Pure Chemical Industry (Osaka, Japan) and used without further purification. Amberlite XAD-7, polymeric absorbent resin was obtained from Organo (Tokyo, Japan).

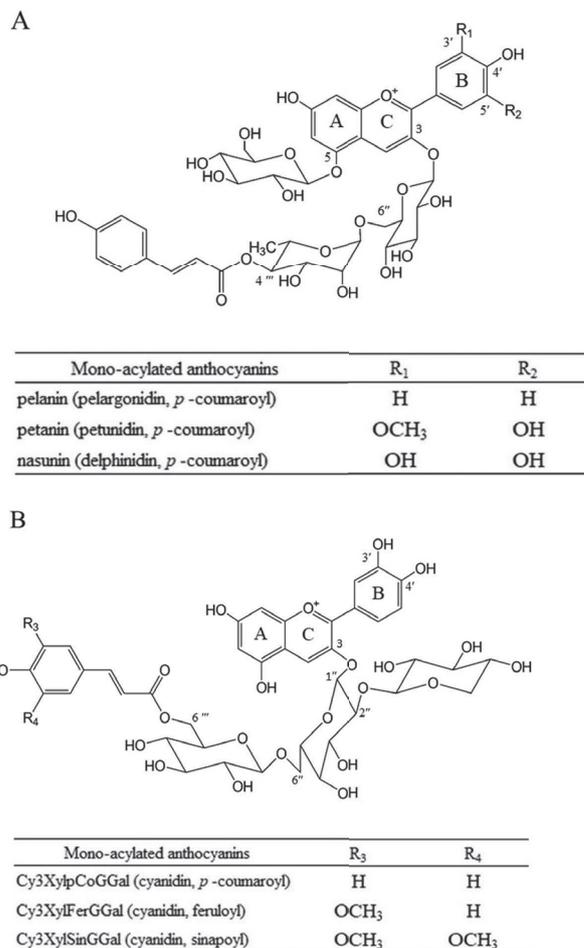


Fig. 1. Structures of Mono-Acylated Anthocyanins

A: mono-acylated anthocyanins derived from colored-fleshed potato and eggplant, **B:** mono-acylated anthocyanins derived from black carrot. Inside of parenthesis show aglycone and acyl moiety of mono-acylated anthocyanins.

Diaion HP-20 resin was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). Sephadex LH-20 (25–100 μm) was obtained from GE Healthcare (NJ, USA). Develosil ODS-HG 5 HPLC columns (1.0 × 250 mm and 20 × 250 mm) were obtained from Nomura Chemical Co., Ltd. (Aichi, Japan). An Aluminum TLC plate, silica gel-coated with fluorescent indicator F₂₅₄ was obtained from Merck Millipore (Darmstadt, Germany).

Plant Materials Anthocyanin-containing red-fleshed potato (Inca-red) and purple-fleshed potato (Inca-purple) (*Solanum tuberosum* L.) were cultivated in the fields of the Potato Breeding Laboratory of the Hokkaido National Agricultural Experiments Station (Memuro, Kasai-Gun, Hokkaido, Japan). Black carrot (*Daucus carota subsp. sativus*) juice was obtained from KAGOME Co. Ltd. (Nagoya, Japan).

Preparation of Colored-Fleshed Potato Extract Inca-red and Inca-purple extracts were obtained as described in a previous study with some modifications.²⁵⁾ Briefly, the fresh tubers of red or purple-fleshed potatoes were sliced to a 1-mm thickness using a slicer, then extracted with 3% TFA aqueous solution overnight at 4°C. After filtration, the pigment extract was passed through an Amberlite XAD-7 column (45 × 600 mm). After washing with distilled water, the absorbed pigments containing anthocyanins were eluted with a 70% ethanol aqueous solution. The extract was then concentrated to dryness *in vacuo*.

uo at 40°C.

Preparation of Black Carrot Extract Black carrot extract was obtained as described in a previous study with some modifications.¹³⁾ Briefly, 1 L of black carrot juice was partitioned with 2 L of ethyl acetate to remove phenolic contaminants in the black carrot. This procedure was repeated four times, after which the water fraction was collected and contaminated ethyl acetate was removed *in vacuo* at 40°C. The water fraction was adsorbed on an HP-20 column (45 × 600 mm), washed with 1% acetic acid aqueous, and the anthocyanin fraction was eluted with 70% aqueous ethanol containing 1% acetic acid and concentrated to dryness *in vacuo* at 40°C.

Purification of Mono-Acylated Anthocyanins Mono-acylated anthocyanins were purified as described in a previous study with modifications.²⁶⁾ Briefly, the extracts of red-fleshed potato, purple-fleshed potato, and black carrot prepared as described above were dissolved in distilled water, after which they were applied to LH-20 column chromatography (30 × 450 mm). Mono-acylated anthocyanins were eluted by stepwise elution under gravity flow with increasing concentrations of methanol in distilled water (0:1→1:0). Fractions containing mono-acylated anthocyanin were collected, evaporated to dryness *in vacuo* at 40°C, and stored in the refrigerator. The anthocyanin-rich fractions were then respectively dissolved in 15% acetic acid aqueous, and mono-acylated anthocyanins in the extracts were isolated by semi-preparative HPLC using a Develosil ODS-HG 5 column (20 × 250 mm) under isocratic elution conditions.²⁶⁾ The mobile phase was a mixture of Solvent A (0.1% TFA aqueous) and Solvent B (0.1% TFA containing acetonitrile) at various ratios [60%A/40%B to 90%A/10%B (v/v)] at a 7 mL/min flow rate, and the effluent was monitored at 520 nm. After the eluent was concentrated to dryness, the isolated pigment was dissolved in a small amount of TFA and precipitated with excess diethyl ether to obtain TFA salts as a reddish-purple powder. The light conversion of (*E*)-*p*-coumaroyl moiety of petanin to (*Z*)-*p*-coumaroyl isomer easily occurred during purification processes as described below, therefore HPLC purification of petanin was carried out under light-shielded conditions. The pigment structure was identified by FAB-MS and NMR spectrometry as described previously.²⁶⁾ The purities of pelanin, petanin, Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal were > 96.2%, > 97.4%, > 98.1%, > 99.2%, and > 89.6%, respectively, as calculated from the peak area of the HPLC chromatogram and the results of TLC detected by UV at 254 nm wave length using ethyl acetate/acetone/acetic acid/water (5:3:1:1, v/v) as developing solvent. Inca-red and Inca-purple potatoes yielded 166 and 184 mg of pelanin and petanin per kg, respectively. Similarly, 1 L of black carrot juice yielded 210, 328, and 138 mg of Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal, respectively.

Conditions for Isomerization Reaction Isomerization of mono-acylated anthocyanins was confirmed according to our previous method.¹⁰⁾ Briefly, five types of mono-acylated anthocyanins were dissolved in 0.1% citric acid aqueous, respectively, and kept under fluorescent light for 4 days at room temperature in a quartz tube. Aliquots of the reaction solution were diluted with 1% TFA aqueous and analyzed by analytical HPLC.

HPLC Analysis HPLC was conducted as described in our previous study with modifications.²⁷⁾ Briefly, aliquots (100 µL) of sample solutions were injected into an HPLC system

(Hitachi L-7200) equipped with a Develosil ODS-HG 5 column (1.0 × 150 mm for administration study and 4.6 × 150 mm for isomerization study) using a linear gradient elution of 0.5% TFA aqueous (Solvent A) and 0.5% TFA-containing acetonitrile (Solvent B) at 40°C. The gradient conditions were as follows: 80% A/20% B (v/v) for 20 min and 55% A/45% B (v/v) for 10 min; 55% A/45% B (v/v) was then held for an additional 10 min at a flow rate of 0.1 mL/min for administration study and 1 mL/min for isomerization study. The elution profile was monitored at 520 nm with a UV-VIS detector (Hitachi L-7000).

Animals and Diets SPF male Wistar ST rats (5-weeks-old, 160 g bodyweight) purchased from Japan SLC, Inc., (Hamamatsu, Japan) were individually housed in stainless-steel wire-mesh cages at 23 ± 1°C for conditioning under a 12-h light/dark cycle. The rats were provided with tap water and a controlled diet *ad libitum* for 7 d prior to the experiment. Animal experiments were performed according to the Guidelines on the Care and Use of Laboratory Animals issued by Niigata University of Pharmacy and Applied Life Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Niigata University of Pharmacy and Applied Life Sciences (Approval number 17-4). All efforts were made to minimize animal suffering.

Experimental Design After 7 d of adaptation, twenty-five rats were cannulated with a polyethylene tube (PE 50) into the neck vein under anesthesia with a mixed solution of medetomidine hydrochloride (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg) by intraperitoneal injection as described in our previous study with modifications.²⁷⁾ Briefly, the neck vein was isolated, and a small hole was cut with scissors to insert the PE 50. Once the tube was inserted, the vein and tube were occluded, and the tube was guided out from the back of the rats. After starving for 24 h, purified anthocyanins dissolved in 0.1% aqueous citric acid were administered orally (100 mg of pelanin, petanin, Cy3XylpCoGGal, Cy3XylFerGGal, or Cy3XylSinGGal/kg body weight) to five rats, respectively. During the experiment, the rats were allowed to move freely in the cages. Blood samples were collected *via* the cannulated tube using a heparinized syringe at 0, 15, 30, 60, 120, 240, and 480 min. Donor blood was collected from other healthy rats under anesthesia by venipuncture of the inferior vena cava into a sodium citrate solution (500 µL of 10% sodium citrate per 8 mL of blood). After the blood was withdrawn (600 µL), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000 × g, 4°C for 5 min to prepare plasma samples for HPLC analysis.

Plasma Preparation Anthocyanins were extracted from blood plasma using a Sep-Pak C₁₈ Light cartridge (Waters, Milford, MA, USA) as reported in our previous study.²⁷⁾ Briefly, plasma (300 µL) samples were applied to Sep-Pak C₁₈ Light cartridges conditioned with methanol (2 mL) and 3% aqueous TFA solution (2 mL). The cartridges were then washed successively with 2 mL of 3% aqueous TFA, dichloromethane, and benzene, and anthocyanins were eluted with 50% aqueous acetonitrile containing 1% TFA. The eluent was concentrated to dryness *in vacuo* and dissolved in 150 µL of 0.5% aqueous TFA. The dissolved sample was passed through a Centri-cut ultra-membrane filter (0.45 µm; Kurabou Co. Ltd., Osaka, Japan) before HPLC injection. The sample recovery of this method was 86.4% for pelanin, 84.8% for petanin, 85.5% for

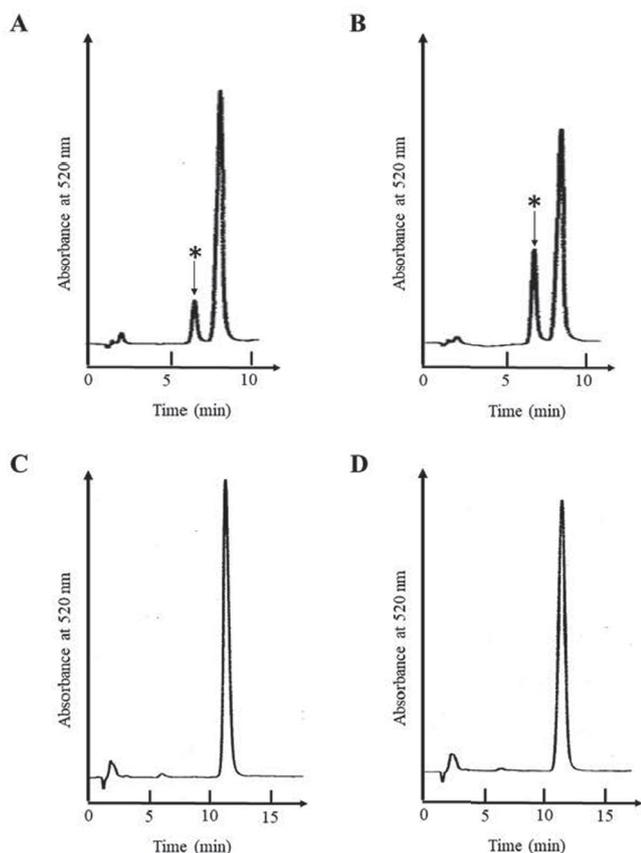


Fig. 2. Typical HPLC Chromatogram of Mono-Acylated Anthocyanins Before and After 72 h Under Fluorescent Light Irradiation Conditions

A: Crude petanin before light irradiation, **B:** Crude petanin 72 h after light irradiation, **C:** Crude Cy3XylpCoGGal before light irradiation, **D:** Crude Cy3XylpCoGGal 72 h after light irradiation. An asterisk indicates (*Z*)-*p*-coumaroyl isomer of petanin.

Cy3XylpCoGGal, 86.7% for Cy3XylFerGGal, and 85.1% for Cy3XylSinGGal, as verified by spiking purified anthocyanins into normal blood plasma.

Statistical Analysis Significant differences among concentrations of anthocyanins in blood plasma were determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$). All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 4.0.3). Concretely, EZR is a modified version of R commander (version 2.7-1) containing statistical functions frequently used in biostatistics.

RESULTS

Fig. 2 shows the HPLC results of mono-acylated anthocyanins before and after 72 h under fluorescent light irradiation. Only, petanin attaching (*E*)-*p*-coumaroyl moiety was exceptionally converted to (*Z*)-*p*-coumaroyl isomer, and the ratio of (*Z*)-*p*-coumaroyl isomer was increased as shown in Fig. 2 B. However, isomerization was not observed for Cy3XylpCoGGal (Fig. 2 C and D). Similar results were obtained for other mono-acylated anthocyanins (pelanin, Cy3XylFerGGal, and Cy3XylSinGGal). Fig. 3A–E shows a typical HPLC chromatogram of purified mono-acylated anthocyanins detected at 520 nm. HPLC analysis revealed that each anthocyanin was absorbed

from the gastrointestinal tract and was present in rat blood plasma in their original mono-acylated forms (Fig. 3F–J). Results clearly demonstrated that (*Z*)-isomers of mono-acylated anthocyanins including petanin were not observed in any HPLC chromatograms of both purified and rat blood plasma samples under light-shielded conditions. However, the contaminant peak observed in purified pelanin was detected in the blood plasma of pelanin-treated rats and exhibited a higher intensity than that of purified pelanin, as shown in Fig. 3F.

Fig. 4 shows the plasma concentration profile of mono-acylated anthocyanins. The maximum plasma concentrations (C_{max}) of mono-acylated anthocyanins were $0.075 \pm 0.008 \mu\text{M}$ for pelanin, $0.342 \pm 0.044 \mu\text{M}$ for petanin, $0.182 \pm 0.031 \mu\text{M}$ for Cy3XylpCoGGal, $0.170 \pm 0.027 \mu\text{M}$ for Cy3XylFerGGal, and $0.187 \pm 0.021 \mu\text{M}$ for Cy3XylSinGGal. The time at which reached the maximum plasma concentration (t_{max}) of petanin, Cy3XylpCoGGal, and Cy3XylFerGGal was 15 min after their oral administration, whereas the t_{max} of pelanin and Cy3XylSinGGal was 30 min. The area under the plasma concentration curve during 8 h after oral administration (AUC_{0-8}) obtained from the plasma concentration profiles of mono-acylated anthocyanins was $15.47 \pm 3.327 \mu\text{M}\cdot\text{min}$ for pelanin, $29.90 \pm 5.114 \mu\text{M}\cdot\text{min}$ for petanin, $13.74 \pm 1.421 \mu\text{M}\cdot\text{min}$ for Cy3XylpCoGGal, $16.45 \pm 0.593 \mu\text{M}\cdot\text{min}$ for Cy3XylFerGGal, and $23.26 \pm 2.777 \mu\text{M}\cdot\text{min}$ for Cy3XylSinGGal (Table 1).

Because the intravenous administration of acylated anthocyanins was not performed in the present study, pharmacokinetic parameters including distribution volume were not determined. Instead of these parameters, the AUC_{0-8} and C_{max} values were normalized to the orally administered dose ($AUC_{0-8}/Dose$ and $C_{max}/Dose$) to compare the gastrointestinal absorption of acylated anthocyanins (Table 1). The $C_{max}/Dose$ values of the mono-acylated anthocyanins were $0.656 \pm 0.074 \mu\text{M}/\text{mmol}/\text{kg}$ for pelanin, $3.140 \pm 0.329 \mu\text{M}/\text{mmol}/\text{kg}$ for petanin, $1.592 \pm 0.088 \mu\text{M}/\text{mmol}/\text{kg}$ for Cy3XylpCoGGal, $1.535 \pm 0.199 \mu\text{M}/\text{mmol}/\text{kg}$ for Cy3XylFerGGal, and $1.746 \pm 0.200 \mu\text{M}/\text{mmol}/\text{kg}$ for Cy3XylSinGGal (Table 1). The $AUC_{0-8}/Dose$ values of the mono-acylated anthocyanins were $135.0 \pm 29.05 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for pelanin, $274.8 \pm 47.00 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for petanin, $120.1 \pm 12.42 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for Cy3XylpCoGGal, $148.7 \pm 5.36 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for Cy3XylFerGGal, and $217.2 \pm 25.94 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for Cy3XylSinGGal (Table 1).

DISCUSSION

The gastrointestinal absorption of mono-acylated anthocyanins has been reported both in experimental animals²¹⁾ and human volunteers^{19,20,22)} using plant materials such as juice or extract as anthocyanin sources. Previous studies have reported that the absorption of non-acylated anthocyanins was several folds higher than that of mono-acylated anthocyanins with aromatic acyl groups.^{19–22)} In contrast, a recent study demonstrated that the plasma concentrations of mono-acylated anthocyanins (Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal) were higher than those of non-acylated anthocyanins that co-occurred in the black carrot extract 60 min after oral administration of the extract in rats.²⁸⁾ These discrepancies suggest that plant components in whole food or juice could influence the gastrointestinal absorption of acylated anthocyanins. Therefore, detailed studies using purified samples are required to clarify the correlation between the structures of acylated anthocyanins and gastrointestinal absorption. Here,

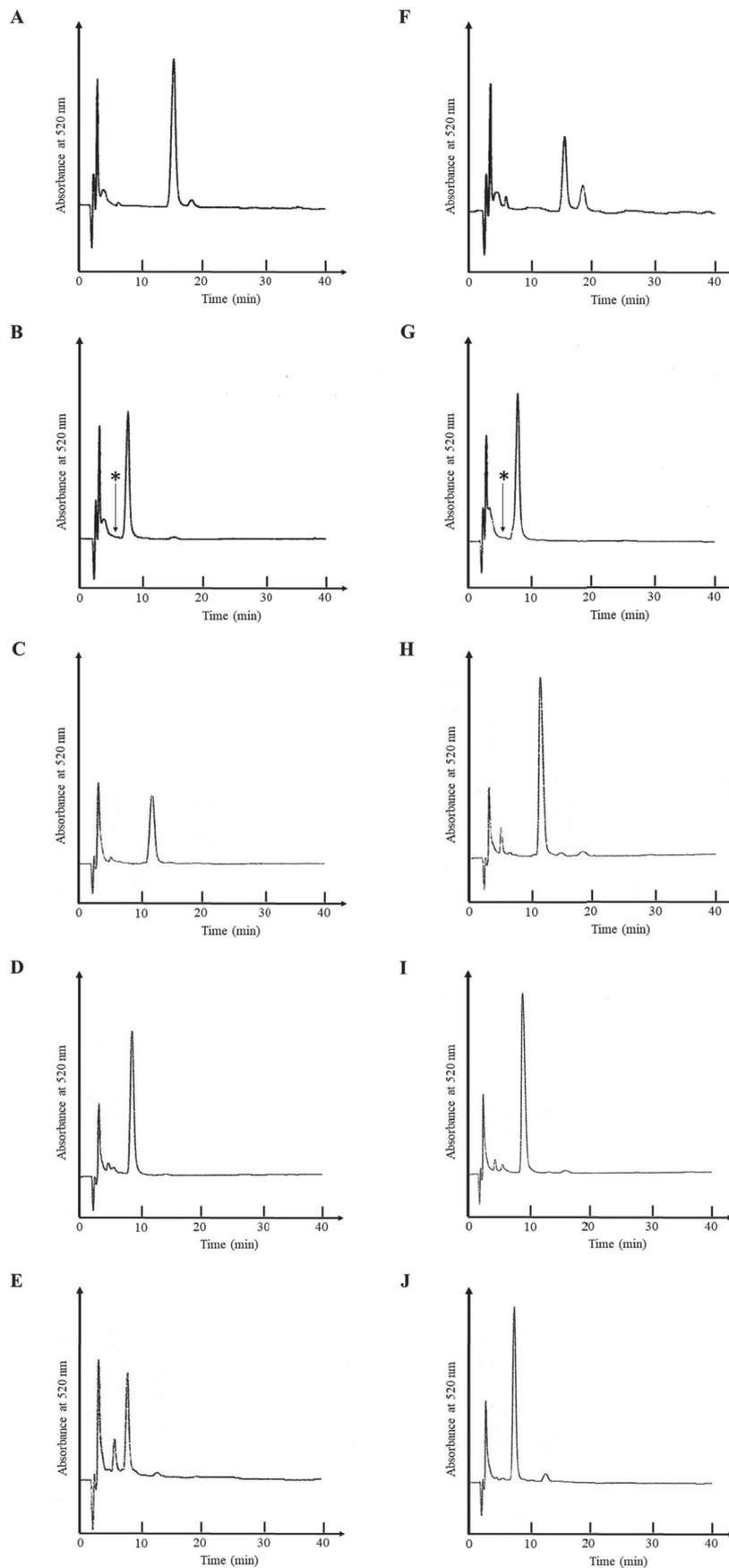


Fig. 3. Typical HPLC Chromatogram of Mono-Acylated Anthocyanins and Rat Blood Plasma

A-E: Purified mono-acylated anthocyanins, A: pelanin, B: petanin, C: Cy3XylpCoGGal, D: Cy3XylFerGGal, E: Cy3XylSinGGal, F-J: rat blood plasma 15 min after the oral administration of purified mono-acylated anthocyanins, F: pelanin, G: petanin, H: Cy3XylpCoGGal, I: Cy3XylFerGGal, J: Cy3XylSinGGal. An asterisk in B and G is corresponding to the retention time of (*Z*)-*p*-coumaroyl isomer of petanin predicted.

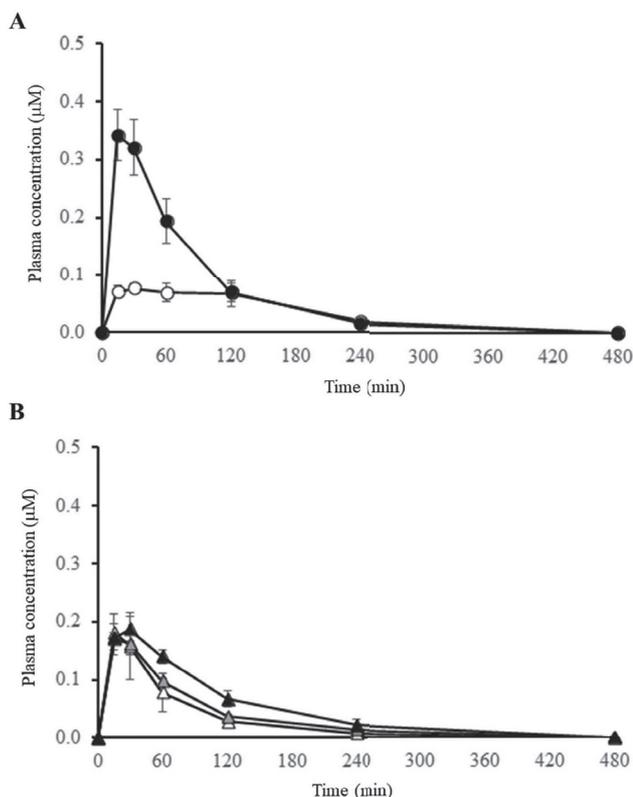


Fig. 4. Plasma Concentration Profiles of Mono-Acylated Anthocyanins After Their Oral Administration

A: Open and closed circles indicate pelanin and petanin, respectively, **B:** Open triangles, gray triangles, and closed triangles indicate Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal, respectively. The values are the means \pm SEM of five rats.

five mono-acylated anthocyanins were purified from colored-fleshed potato and black carrot, and their gastrointestinal absorption was evaluated based on plasma concentration profiles such as $AUC_{0-8}/Dose$ and $C_{max}/Dose$ in rats. Furthermore, mono-acylated anthocyanins were classified into two types depending on the patterns of acylated sugars to evaluate the correlations between mono-acylated anthocyanin structure and absorption: anthocyanidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*- β -D-glucopyranoside (pelanin, petanin, and nasunin) attaching non-branched acylated sugars as type A mono-acylated anthocyanins and anthocyanidin 3-*O*-(2''-xylopyranosyl-6''-*O*-(6'''-*O*-acyl- β -D-glucopyranosyl))- β -D-galactopyranoside (Cy3X-

ylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal) attaching branched acylated sugars as type B mono-acylated anthocyanins.

Anthocyanins including mono-acylated derivatives are known as hydrophilic polyphenol components present in various plant materials.¹⁾ In the present study, the solubility of acylated anthocyanins was checked visually, as a relatively high concentration of mono-acylated anthocyanin solution (20 mg/mL) was prepared for administration study. As a result, undissolved anthocyanins were not observed in any mono-acylated anthocyanin solution, therefore, it was confirmed that mono-acylated anthocyanins were well dissolved in 0.1% citric acid aqueous, partially in the flavylium cation forms, up to 20 mg/mL concentration.

We have previously reported that the (*E*)-*p*-coumaroyl moiety of nasunin could be easily converted to (*Z*)-*p*-coumaroyl isomer under fluorescent light conditions.¹⁸⁾ Therefore, the isomerization of five types of mono-acylated anthocyanins was confirmed under fluorescent light. Results demonstrated isomerization of four out of five mono-acylated anthocyanins was not observed under fluorescent light except petanin. Therefore, the final purification steps of petanin by semi-preparative HPLC were carried out under light-shielded conditions. Also, all of the animal experimental procedures including extraction of anthocyanin from blood plasma were carried out under light-shielded conditions as described in our previous report¹⁸⁾ to avoid the artificial isomerization of mono-acylated anthocyanins during the experiment. HPLC results clearly demonstrated (*Z*)-*p*-coumaroyl isomers were not observed in any mono-acylated anthocyanins both in the purified samples and mono-acylated anthocyanin administered rat blood plasma under light-shielded conditions.

Our HPLC results demonstrated that the petanin used in the administration study was sufficiently pure, whereas pelanin exhibited a contamination peak. Petanin was absorbed in rat blood plasma in its original mono-acylated form 15 min after oral administration, which was similar to the results of a previous study using nasunin.¹⁸⁾ The results clearly demonstrated that the plasma concentration profile of petanin ($274.8 \pm 47.00 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for $AUC_{0-8}/Dose$ and $3.140 \pm 0.329 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{max}/Dose$) was similar to those reported in a previous nasunin study ($280.2 \pm 38.62 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for $AUC_{0-8}/Dose$ and $3.082 \pm 0.365 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{max}/Dose$)¹⁸⁾ and slightly higher than those of the non-acylated anthocyanin Dp3G ($255.3 \pm 39.94 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for $AUC_{0-8}/Dose$ and $1.346 \pm 0.297 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{max}/Dose$).¹⁸⁾ Nasunin and petanin respectively carry delphinidin and petuni-

Table 1. Dose and Plasma Parameters of Orally Administered Mono-Acylated Anthocyanins

Mono-acylated anthocyanin	C_{max} (μM)	t_{max} (min)	AUC ($\mu\text{M}\cdot\text{min}$)	$Dose$ (mmol/kg)	$C_{max}/Dose$ ($\mu\text{M}/\text{mmol}/\text{kg}$)	$AUC/Dose$ ($\mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$)
pelanin (pelargonidin, <i>p</i> -coumaroyl)	$0.075 \pm 0.008^{a,b}$	30	15.465 ± 3.327^f	0.1145	$0.656 \pm 0.074^{h,i}$	$135.01 \pm 29.05^{p,q}$
petanin (petunidin, <i>p</i> -coumaroyl)	$0.342 \pm 0.044^{a,c,d,e}$	15	$29.898 \pm 5.114^{e,g}$	0.1088	$3.140 \pm 0.329^{h,j,k,l}$	$274.77 \pm 47.00^{p,r}$
nasunin (delphinidin, <i>p</i> -coumaroyl)*	0.292 ± 0.042^b	15	26.611 ± 3.657	0.0947	$3.082 \pm 0.365^{i,m,n,o}$	$280.19 \pm 38.62^{q,s,t}$
Cy3XylpCoGGal (cyanidin, <i>p</i> -coumaroyl)	0.182 ± 0.031^c	15	13.744 ± 1.421^e	0.1144	$1.592 \pm 0.088^{j,m}$	$120.12 \pm 12.42^{r,s}$
Cy3XylFerGGal (cyanidin, feruloyl)	0.170 ± 0.027^d	15	16.449 ± 0.593	0.1106	$1.535 \pm 0.199^{k,n}$	148.70 ± 5.36^t
Cy3XylSinGGal (cyanidin, sinapoyl)	0.187 ± 0.021^e	30	23.256 ± 2.777	0.1071	$1.746 \pm 0.200^{l,o}$	217.21 ± 25.94

C_{max} , maximum plasma concentration; t_{max} , time at which the maximum plasma concentration was reached; AUC_{0-8} , area under the plasma concentration curve during the 8-h period after oral administration.

*Data cited from reference 18 for comparison.

The values represent the means \pm SEM of five rats.

Values with the same lowercase letters indicate a significant difference ($P < 0.05$).

Inside of parenthesis show aglycone and acyl moiety of mono-acylated anthocyanins.

din (3'-*O*-methyl analog of delphinidin) as aglycone moieties, and therefore our findings indicated that the substitution of the *O*-methyl group on position 3' of delphinidin did not influence the intestinal absorption of type A mono-acylated anthocyanins.

In contrast, the plasma profiles of pelanin ($135.0 \pm 29.05 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $0.656 \pm 0.074 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$) were significantly lower than those of petanin ($274.8 \pm 47.00 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $3.140 \pm 0.329 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$) and nasunin ($280.2 \pm 38.62 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $3.082 \pm 0.365 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$)¹⁸⁾ ($P < 0.05$). Exceptionally high plasma profiles of pelargonidin 3-*O*- β -D-glucopyranoside (Pg3G) have been reported both in rats^{6,29)} and humans.³⁰⁾ Furthermore, we previously reported that the absorption of Pg3G in its original form ($483.9 \pm 49.58 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $5.867 \pm 0.882 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$)⁶⁾ was higher than other non-acylated anthocyanins such as Cy3G ($108.9 \pm 33.65 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $0.820 \pm 0.113 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$)⁴⁾ or Dp3G ($255.3 \pm 39.94 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $1.346 \pm 0.297 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$)¹⁸⁾. However, the plasma levels of pelargonidin 3-*O*- β -D-glucuronide (Pg3GlcA) ($1213.4 \pm 335.5 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $7.423 \pm 0.929 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$), the dominant metabolite of Pg3G, were more than 2-fold higher than those of Pg3G.⁶⁾ In this study, Pg3GlcA was not produced as a major metabolite of pelanin. For the production of Pg3GlcA, intestinal hydrolysis of the acylated sugar moiety of pelanin is required. A recent report demonstrated that Cy3XylFerGGal isolated from black carrot exhibited limited hydrolysis of acylated sugars, indicating that further glucuronidation of anthocyanidin did not occur for mono-acylated anthocyanins during the absorption processes in the small intestine.²⁸⁾ Therefore, the lower absorption of pelanin compared with Pg3G (i.e., a non-acylated anthocyanin) was attributed to the limited pelanin acyl hydrolysis and did not undergo successive glucuronidation of released pelargonidin. Interestingly, pelanin also exhibited exceptionally poor absorption profiles in its original form compared to the other mono-acylated anthocyanins examined here. This suggests that the entire structural balance of mono-acylated anthocyanins such as the combinations of aglycone and attached aromatic acylated sugars influence their gastrointestinal absorption. On the other hand, a contaminant peak for which structural information was not obtained was detected in both the purified pelanin and in rat blood plasma; however, this peak was more prominent in the plasma samples than in the purified pelanin indicating higher absorption of contaminant than pelanin. Because the contaminant detected at 520 nm wave length was predicted as anthocyanin, poor absorption of pelanin may be explained by the molecular competition on the intestinal transporter between pelanin and the contaminant anthocyanin as described in the section below.

The purities of the mono-acylated anthocyanins isolated from black carrots were sufficiently high, with only Cy3XylSinGGal exhibiting relatively low purity. However, unlike the pelanin results, contaminant peaks were not detected in rat blood plasma. The AUC_{0-8}/Dose values of type B mono-acylated anthocyanins isolated from black carrots (Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal) were higher than those of Cy3G, a non-acylated anthocyanin with the same aglycones ($108.9 \pm 33.65 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$)⁴⁾ Upon comparing

the absorption of the different type B mono-acylated anthocyanins, Cy3XylSinGGal exhibited the highest plasma profiles ($217.2 \pm 25.94 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $1.746 \pm 0.200 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$), followed by Cy3XylFerGGal ($148.7 \pm 5.36 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $1.535 \pm 0.199 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$) and Cy3XylpCoGGal ($120.1 \pm 12.42 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ for AUC_{0-8}/Dose and $1.592 \pm 0.088 \mu\text{M}/\text{mmol}/\text{kg}$ for $C_{\text{max}}/\text{Dose}$). Therefore, the amount of intestinal absorption of mono-anthocyanins is not strictly correlated with the extent of the methoxyl group of acyl moiety.

As described in the section above, we classified mono-acylated anthocyanins into two groups depending on the attached patterns of acylated sugars to discuss the structural effect of mono-acylated anthocyanin on gastrointestinal absorption. Concretely, type A mono-acylated anthocyanins exhibit non-branched acylated sugars (6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranosyl moiety), whereas type B mono-acylated anthocyanins contain branched acylated sugars (2''-xylopyranosyl-6''-*O*-(6'''-*O*-acyl- β -D-glucopyranosyl)- β -D-galactopyranosyl moiety) in their structure, as shown in Fig. 1. The AUC_{0-8}/Dose levels of the type A mono-acylated anthocyanins nasunin ($280.19 \pm 38.62 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$)¹⁸⁾ and petanin ($274.77 \pm 47.00 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$) were 1.3 to 2.3 fold higher than those of type B mono-acylated anthocyanins derived from black carrot (Cy3XylpCoGGal, Cy3XylFerGGal, and Cy3XylSinGGal) (statistically significant differences were identified between petanin and Cy3XylpCoGGal, nasunin and Cy3XylpCoGGal, and nasunin and Cy3XylFerGal; $P < 0.05$). Therefore, the present results lead to the hypothesis that the absorption amounts of type A mono-acylated anthocyanins with non-branched acylated sugars except for pelanin could be higher than those of type B mono-acylated anthocyanins with branched acylated sugars. Because the relative ratios of acylated sugars in the whole molecular structure of mono-acylated anthocyanins were generally high, attached patterns of acylated sugars may be the dominant modulator of gastrointestinal absorption of mono-acylated anthocyanins. However, to prove this hypothesis, the absorption of type A mono-acylated anthocyanins attaching cyanidin as aglycone (cyananin: cyanidin 3-*O*-(6''-*O*-(4'''-*O*-*p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)-5-*O*- β -D-glucopyranoside) must be evaluated to compare with that of type B mono-acylated anthocyanin, Cy3XylpCoGGal. Also, the effect of aglycones and aromatic acyl groups must be taken into account to evaluate intestinal absorption of mono-acylated anthocyanins as was demonstrated in the case of pelanin and Cy3XylSinGGal.

The AUC_{0-8}/Dose values of mono-acylated anthocyanins ($120.1 \pm 12.42 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$ to $274.8 \pm 47.00 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$) examined in the present study except pelanin ($135.0 \pm 29.05 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$) were slightly higher or at least similar to those of the non-acylated anthocyanins Dp3G ($255.3 \pm 39.94 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$)¹⁸⁾ or Cy3G ($108.9 \pm 33.65 \mu\text{M}\cdot\text{min}/\text{mmol}/\text{kg}$)⁴⁾ We have previously reported that the absorption amounts of nasunin and ternatin were similar or slightly higher than those of the non-acylated anthocyanin Dp3G using purified or semi-purified anthocyanins.^{18,27)} Furthermore, we recently demonstrated that the attached malonyl groups in the glucopyranosyl moiety of cyanidin enhanced the AUC_{0-8}/Dose values of anthocyanin using semi-purified anthocyanins obtained from the petals of Japanese edible red chrysanthemum.

mum (*Dendranthema grandiflorum*): cyanidin 3-*O*- β -D-(3", 6"-di-*O*-malonyl)-glucopyranoside > cyanidin 3-*O*- β -D-(6"-mono-*O*-malonyl)-glucopyranoside > Cy3G.³¹⁾ Taken together, our present and previous studies revealed that the absorption amounts of acylated anthocyanins were generally higher or at least similar to those of non-acylated anthocyanins when purified or semi-purified anthocyanins from which hydrophilic components such as sugars and organic acid were removed were orally administered, and this phenomenon did not depend on the aromatic or aliphatic acyl groups attached to the acylated anthocyanins.

In contrast to our results, other studies have reported that the absorption of mono-acylated anthocyanins was lower than that of non-acylated anthocyanins that co-occurred in plant materials when black carrot,¹⁹⁾ red cabbage²⁰⁾ and purple-fleshed sweet potato²²⁾ (both in juice or whole) were used as anthocyanin sources. A previous study reported that non-acylated anthocyanins were transported by both organic anion transporter peptide (OATP)²⁸⁾ and glucose transporter-2 (GLUT-2)³²⁾ in the gastrointestinal tract, whereas acylated anthocyanins were transported by OATP alone.²⁸⁾ Therefore, it was predicted that the absorption of mono-acylated anthocyanins via OATP was competitively inhibited by non-acylated anthocyanins or organic acids with a strong affinity for OATP that were present in juice or whole foods, in turn, resulting in the poor absorption of mono-acylated anthocyanins than non-acylated anthocyanins which were also transported via GLUT-2. In contrast, mono-acylated anthocyanins were effectively transported by OATP when the purified mono-acylated anthocyanins from which both organic acid and non-acylated anthocyanins were removed were orally administered leading to the result that mono-acylated anthocyanins were absorbed in similar or higher levels than non-acylated anthocyanins. This suggests that the transportation of mono-acylated anthocyanins via OATP was more effective than that of non-acylated anthocyanins via both GLUT-2 and OATP routes. Additionally, the relatively poor absorption of type B mono-acylated anthocyanins compared to type A mono-acylated anthocyanins may be explained by differences in the recognition of branched and non-branched acylated sugars by OATP. Further studies are required to assess the effects of the competition among acylated anthocyanins, non-acylated anthocyanins, and organic acids on absorption mechanisms via intestinal transporters such as OATP using purified anthocyanins.

In conclusion, this was the first to evaluate the relationship between the structures and gastrointestinal absorption of mono-acylated anthocyanin using purified samples. The gastrointestinal absorption of mono-acylated anthocyanins with aromatic acyl groups may be modulated by the structures of the attached acylated sugars. Branched acylated sugars attached to position 3 of aglycone possibly could suppress the absorption of acylated anthocyanins from the gastrointestinal tract, although, the effects of aglycone and aromatic acyl groups were exceptionally observed in a few cases. Furthermore, using purified samples, results demonstrated that the absorption of mono-acylated anthocyanins was generally higher than that of non-acylated anthocyanins and that this did not depend on the branched or non-branched acylated sugars attached to the tested compounds. Additionally, the poor absorption of mono-acylated anthocyanins from whole foods or juice reported previously could be explained by the competition of OATP transport with organic acids co-occurring

in plant materials. Collectively, our findings provide basic insights into the health-promoting effects of mono-acylated anthocyanins in biological systems.

Conflict of interest The authors declare no conflict of interest.

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