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Identification of Reaction Products of Acylated Anthocyanins from Red Radish with Peroxyl Radicals

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Red radish anthocyanin extract, which consists of 12 known acylated anthocyanins, was reacted with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) to generate peroxyl radicals under acidic pH conditions at 37 °C. The reaction products were isolated using preparative HPLC, and their chemical structures were determined to be *p*-hydroxybenzoic acid (**1**), 6-*O*-(*E*)-*p*-coumaroyl-2-*O*-β-D- glucopyranosyl-α-D-glucopyranoside (**3**), *p*-coumaric acid (**4**), 6-*O*-(*E*)-feruloyl-2-*O*-β-D-glucopyranosyl-α-D-glucopyranoside (**5**), and ferulic acid (**6**). Some products were not identified. HPLC analyses of the mixture of acylated pelargonidin isolated from red radish and AAPH revealed that the acylated pelargonidins possess the radical scavenging ability on some common sites even if the characteristics of the intramolecular acyl units are different. Degradation rates of acylated pelargonidins and the formation rates of the resulting reaction products were found to be quite different.

KEYWORDS: Acylated anthocyanin; pelargonidin; peroxyl radical; AAPH; oxidation product

INTRODUCTION

Anthocyanins are an important group of dietary antioxidants, which are relatively abundant in vegetables and fruits. Anthocyanins scavenge free radicals such as peroxyl, superoxide anion, and hydroxyl radicals (*1*−*7*), and suppress lipid and low-density lipoprotein oxidation induced by Cu^{2+} or Fe^{2+} (*8*−*10*). Also, anthocyanins are found to be absorbed in their intact, naturally occurring glycoside forms (*11*−*15*). These findings suggest that anthocyanins would act as an antioxidant in vivo. Recently, dietary antioxidants have attracted considerable interest due to the beneficial effects for the maintenance of health and prevention of various diseases such as cancer, cardiovascular diseases, arthritis, and diabetes (*16*−*18*). The physiological function of dietary antioxidants is suggested to protect living organisms from oxidative damage, resulting in the prevention of diseases.

The anthocyanidins (aglycones) are characterized by having the flavylium (2-phenylbenzopyrylium) cation structure and different hydroxyl or methoxyl substitutions on the B-ring. Although many studies concerning structure-antioxidant activity relationships of aglycones and the glycosides have been done, literature on the evaluation of antioxidant potency of anthocyanins is confusing and difficult to interpret because different testing systems and methods are used for oxidation determination (*19*). Tsuda et al. (*20*) examined the reaction products of anthocyanin and alkylperoxyl radical in detail, and concluded that 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzenofuran and protocatechuic acid were produced from cyanidin 3-*O*-β-D-glucoside. The resulting protocatechuic acid was also found to possess antioxidant activity. In addition, they reported that *p*-hydroxybenzoic acid was produced by the reaction of pelargonidin and peroxinitrite, and that the resulting *p*-hydroxybenzoic acid also scavenged peroxynitrite (*21*).

The natural occurring anthocyanins are glycosides, and acylated forms are frequently found. Acylation makes the anthocyanins more stable through intramolecular copigmentation (*22*−*24*). Thus, the acylated anthocyanins are worthy of attention because they are potential natural alternatives to artificial colorants. In addition, they show stronger antioxidant activity than those of aglycones and the glycosides because anthocyanin and intramolecular acyl units such as caffeoyl, feruloyl, and *p*-coumaroyl groups act synergistically (*25*−*27*). However, the antioxidant mechanism and effects of the acylated anthocyanin are obscure because acylated anthocyanins are not commercially available and must be extracted from vegetables and fruits.

The red radish (*Raphanus sativus* L.) is widely used as a vegetable and for natural food colors. It contains a significant amount of anthocyanins, the major components being acylated pelargonidin glycosides with a combination of *p*-coumaric, ferulic, or malonic acids (*28*, *29*). In our previous report (*30*), 12 acylated anthocyanins with a combination of caffeic, ferulic, or *p*-coumaric acids, containing six novel compounds, were isolated from red radish (**Figure 1**).

To understand the mechanism of antioxidant activity of acylated anthocyanins, it is important to analyze the oxidation products that may arise during its action as an antioxidant. In this study, we attempted to isolate and characterize the reaction

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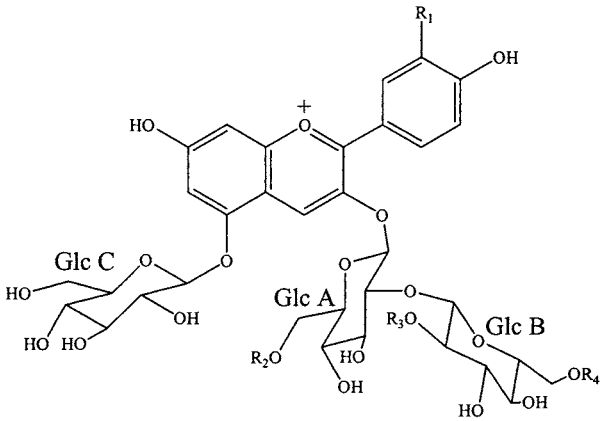
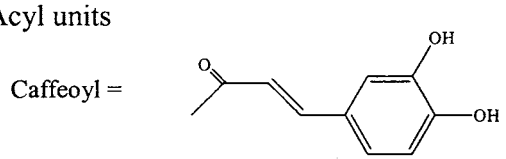
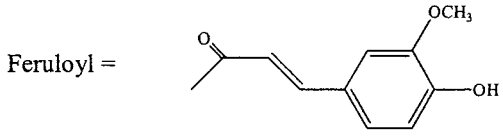
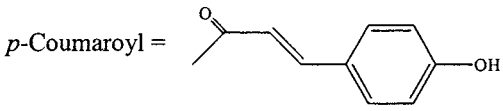


Acyl units







RRA-1	R_1=H	R_2=H	R_3=H	R_4=Feruloyl
RRA-2	R_1=OH	R_2=Caffeoyl	R_3=H	R_4=Feruloyl
RRA-3	R_1=H	R_2=Caffeoyl	R_3=H	R_4=H
RRA-4	R_1=H	R_2=Caffeoyl	R_3=H	R_4=Caffeoyl
RRA-5	R_1=H	R_2=Caffeoyl	R_3=H	R_4=Feruloyl
RRA-6	R_1=H	R_2=*p*-Coumaroyl	R_3=H	R_4=H
RRA-7	R_1=H	R_2=*p*-Coumaroyl	R_3=H	R_4=Caffeoyl
RRA-8	R_1=H	R_2=Feruloyl	R_3=H	R_4=H
RRA-9	R_1=H	R_2=Feruloyl	R_3=H	R_4=Caffeoyl
RRA-10	R_1=H	R_2=*p*-Coumaroyl	R_3=H	R_4=Feruloyl
RRA-11	R_1=H	R_2=Feruloyl	R_3=H	R_4=Feruloyl
RRA-12	R_1=H	R_2=Feruloyl	R_3=Feruloyl	R_4=H

Figure 1. Chemical structures of anthocyanins isolated from red radish.

products of acylated anthocyanin and peroxyl radicals generated by the thermolysis of a free radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH).

MATERIALS AND METHODS

Materials. Red radish anthocyanin extract was obtained after a single purification step of red radish color (San-Ei Gen F. F. I. Inc., Osaka, Japan) by a Diaion HP-20 column chromatography (70% methanol fraction), as described in our previous report (*30*). The extract consisted of the 12 acylated anthocyanins, RRA-1 (0.9%), RRA-2 (0.3%), RRA-3 (2.3%), RRA-4 (2.5%), RRA-5 (7.1%), RRA-6 (10.2%), RRA-7 (3.4%), RRA-8 (17.8%), RRA-9 (10.8%), RRA-10 (8.6%), RRA-11 (24.5%), and RRA-12 (2.2%) (**Figure 1**). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of HPLC or special grade and purchased from Wako Chemical Ind. (Osaka, Japan) and were used without further purification. DMSO-d_6 and TFA-d_1 were obtained from Isotech Inc. (Miamisburg, OH).

Isolation of Reaction Products. Red radish anthocyanin extract (140 mg) dissolved in 4.0 mL of MeOH containing 0.1% TFA was incubated with AAPH (80 mg; 300 μmol) in 100 mmol/L Tris solution adjusted to pH 3 with HCl (8.0 mL) at 37 °C in darkness for 4 days. The pH of this reaction solution was about 2.8. An aliquot of the mixture was taken for LC/MS. The reaction mixture was subjected to preparative HPLC by a 250 × 10 mm i.d. Capcell Pak C18 column (Shiseido Fine Chemicals, Tokyo, Japan) using a linear MeOH gradient (12−20%, 40 min) in 1% HCOOH. After 40 min, the mobile phase was changed to 95% MeOH containing 1% HCOOH so as to remove unreacted red radish anthocyanins. Flow rate was maintained at 5 mL/min, and the effluent was monitored at 300 nm.

LC/MS Analysis. An AQA mass spectrometer (Thermoquest, Manchester, UK) equipped with an atomospheric pressure chemical ionization (APCI) source and coupled to a HP1100 series HPLC with a photodiode array detector (Hewlett-Packard, Tokyo, Japan) was used for LC/MS analysis. The reaction mixture was injected onto a 250 × 4.6 mm i.d. Capcell Pak C18 ACR column, and eluted with a linear MeOH gradient in 0.1% HCOOH (12−20% MeOH in 40 min and 20−95% in 10 min) at a flow rate of 1.0 mL/min at 40 °C. The APCI

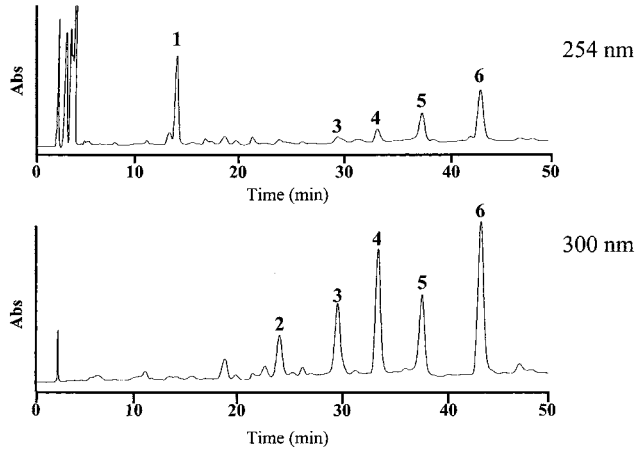


Figure 2. HPLC profiles of the reaction products of red radish anthocyanin extract with AAPH.

probe voltage and the capillary temperature were maintained at 3.0 kV and 200 °C, respectively. The mass spectrometer was operated in the positive ion mode, with a scan range from *m*/*z* 200 to 1200.

Spectroscopic Analysis. High-resolution FAB mass spectra were analyzed by a JMS-SX 102A mass spectrometer (JEOL, Tokyo, Japan) with glycerol as a matrix in the positive mode. ^{1}H- and ^{13}C NMR spectra were measured at 600 and 150 MHz, respectively, in a mixed solvent of DMSO-d_6 and TFA-d_1 (9:1) with tetramethylsilane as an internal standard. The signals in the ^{1}H and ^{13}C NMR spectra of the isolated reaction products were assigned on the basis of chemical shifts and the results of heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) studies.

Time Course of the Formation of Reaction Products. The acylated anthocyanin (0.1 μmol) isolated from red radish was dissolved in 0.5 mL of MeOH containing 0.1% TFA, then incubated with AAPH (0.5 μmol) in 100 mmol/L Tris solution adjusted to pH 3 with HCl (1.0 mL) at 37 °C. Small aliquots of each mixture were withdrawn periodically for analysis of the reaction products and residual acylated anthocyanin by the reverse-phase HPLC system with a 250 × 4.6 mm i.d. Capcell Pak C18 ACR column and a photodiode array detector. The following conditions were used: solvent, 12−20% MeOH in 1% HCOOH (0−40 min), then 20−95% MeOH in 1% HCOOH (40−180 min); flow rate, 1.0 mL/min; column temperature, 40 °C. The experiment was replicated three times.

RESULTS AND DISCUSSION

Figure 2 shows the HPLC profiles of the reaction mixture between red radish anthocyanin extract and AAPH. The reaction resulted in the formation of six novel products (**1**, 13 min; **2**, 23 min; **3**, 29 min; **4**, 32 min; **5**, 38 min; and **6**, 42 min). Some minor peaks were also detected. The diode array spectra revealed that absorption maxima of these compounds (**1**, **2**, **3**, **4**, **5**, and **6**) were at 254, 295, 310, 310, 325, and 325 nm, respectively. When the reaction mixture was analyzed by HPLC at intervals of 1 day, the formation of these six peaks reached the maximum value after 4 days (data not shown). In addition, the formation of these peaks under the condition without AAPH was not observed. The compounds were collected by preparative HPLC and from about 140 mg of red radish anthocyanin extract, 5 mg of **1**, 2 mg of **3**, 4 mg of **4**, 3 mg of **5**, and 5 mg of **6** were obtained. Since compound **2** was obtained in insufficient amounts, **1**, **3**, **4**, **5**, and **6** were used for further examinations.

The structure of **1** was identified as *p*-hydroxybenzoic acid (**Figure 3**). The APCI-LC/MS spectrum showed the molecular ion at *m*/*z* 138 $[M]^+$ corresponding to $C_7H_6O_3$. The co-injection analysis of **1** with authentic *p*-hydroxybenzoic acid by HPLC confirmed the structure.

The structures of **4** and **6** were identified as *p*-coumaric acid and ferulic acid, respectively (**Figure 3**). The APCI-LC/MS data of **4** and **6** showed molecular ion related peaks at *m*/*z* 164 $[M]^+$ and 146 $[M - H_2O]^+$ ($C_9H_8O_3$) and *m*/*z* 194 $[M]^+$ and 176 $[M - H_2O]^+$ ($C_{10}H_{10}O_4$), respectively. The ^{1}H- and ^{13}C NMR analyses and co-injection analyses of **4** and **6** with authentic compounds by HPLC confirmed these structures.

Compound **3** showed UV−vis maxima at λ 294 nm (ϵ 23 400) and 310 nm (ϵ 25 000) in MeOH. In the APCI-LC/MS spectrum of **3**, the molecular ion and fragment ions at *m*/*z* 488 $[M]^+$, 470 $[M - H_2O]^+$, 164, and 146 were observed. High-resolution mass spectrometry gave the protonated molecular formula as $C_{21}H_{29}O_{13}$ ($[M + H]^+$ 489.1624, calcd 489.1608). The ^{1}H NMR data suggested that **3** had a *p*-coumaroyl unit and two sugar units, as seen by the four proton signals from the *p*-coumaroyl unit (δ 6.42, 6.82, 7.58, and 7.59) and the two anomeric proton signals (δ 4.32 and 5.20) for the sugar units. The coupling constants of the anomeric proton signals at δ 4.32 and 5.20 were 7.5 and 3.0 Hz, respectively, thus suggesting that one glucose unit was of β-D-glucopyranoside type and the other was of α-D-glucopyranoside type. In the HMBC spectra, correlations between the anomeric proton signal at δ 4.32 (assigned to the 1-position of glucose B) and the carbon signal at δ 81.9 (assigned to the 2-position of glucose A), and between the proton signals at δ 4.20 and 4.32 (assigned to the 6-position of glucose A) and the carbon signal at 166.4 (assigned to the

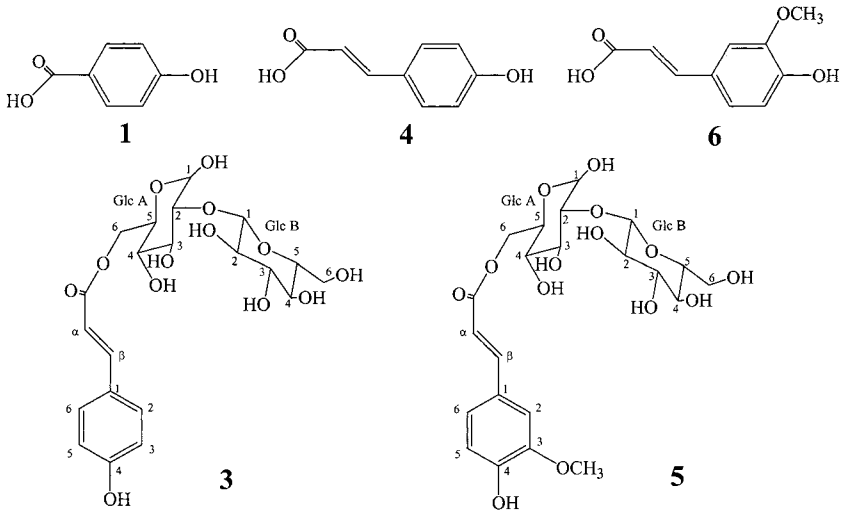


Figure 3. Chemical structures of the reaction products of red radish anthocyanin extract with AAPH.

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for Reaction Products in DMSO-d_6 and TFA-d_1 (9:1)

	^{1}H		^{13}C	
	3	**5**	**3**	**5**
acyl unit				
1			125.2	125.7
2	7.59 (2H, *d*, *J* = 8.0 Hz)	7.35 (1H, *br s*)	130.3	111.2
3	6.82 (2H, *d*, *J* = 8.0 Hz)		115.8	148.0
4			159.8	149.4
5		6.83 (1H, *d*, *J* = 8.0 Hz)		115.6
6		7.15 (1H, *br d*, *J* = 8.0 Hz)		123.3
α	6.42 (1H, *d*, *J* = 16.0 Hz)	6.51 (1H, *d*, *J* = 16.0 Hz)	114.2	114.5
β	7.58 (1H, *d*, *J* = 16.0 Hz)	7.57 (1H, *d*, *J* = 16.0 Hz)	145.0	145.2
OMe		3.84 (3H, *s*)		55.8
carbonyl			166.4	166.8
Glc A				
1	5.20 (1H, *d*, *J* = 3.0 Hz)	5.21 (1H, *d*, *J* = 2.5 Hz)	91.5	91.7
2	3.27 (1H, *dd*, *J* = 3.0, 9.5 Hz)	3.29 (1H, *dd*, *J* = 2.5, 9.5 Hz)	81.9	82.0
3	3.70 (1H, *m*)	3.71 (1H, *m*)	71.4	71.6
4	3.22 (1H, *t*, *J* = 9.5 Hz)	3.25 (1H, *t*, *J* = 9.5 Hz)	70.0	70.2
5	3.88 (1H, *m*)	3.88 (1H, *m*)	69.1	69.2
6a	4.20 (1H, *dd*, *J* = 6.0, 11.5 Hz)	4.22 (1H, *dd*, *J* = 6.0, 11.5 Hz)	63.6	63.8
6b	4.38 (1H, *br d*, *J* = 11.5 Hz)	4.38 (1H, *d*, *J* = 11.5 Hz)		

carbonyl carbon of *p*-coumaric acid) were observed. Thus, **3** was determined to be 6-*O*-(*E*)-*p*-coumaroyl-2-*O*-β-D-glucopyranosyl-α-D-glucopyranoside (**Figure 3**). The ^{1}H and ^{13}C NMR data are presented in **Table 1**.

Compound **5** showed UV−Vis maxima at λ 295 nm (ε 14 800) and 320 nm (ε 17 600) in MeOH. In the APCI-LC/MS spectrum of **5**, peaks at *m*/*z* 518 $[M]^+$, 500 $[M - H_2O]^+$, 194, and 176 were observed. High-resolution mass spectrometry gave the protonated molecular formula as $C_{22}H_{31}O_{14}$ ($[M + H]^+$ 519.1719, calcd 519.1713). The ^{1}H NMR data suggested that **5** had a feruloyl unit and two sugar units. Also, the data for the two sugar units were nearly the same as that of **3**. The HMBC correlations between the proton signal at 6-position of glucose A and the carbonyl carbon signal of feruloyl unit and between the anomeric proton signal of glucose B and the carbon signal at the 2-position of glucose A were observed. Thus, **5** was determined to be 6-*O*-(*E*)-feruloyl-2-*O*-β-D-glucopyranosyl-α-D-glucopyranoside (**Figure 3**). The ^{1}H and ^{13}C NMR data are presented in **Table 1**.

To investigate the distributions of reaction products and their relative formation rates, incubation was carried out with the isolated acylated anthocyanin from red radish and AAPH. In this experiment, we used three acylated anthocyanins, RRA-5, RRA-9, and RRA-11. **Figure 4** shows the HPLC profiles of the reaction mixture between the acylated anthocyanins and AAPH. Some peaks (**1**, **2**, **5**, and **6**) were identified by their retention times and the UV−vis absorption spectra of the photodiode array detector. Interestingly, these HPLC profiles were different from those of the mixture of red radish anthocyanin extract and AAPH (**Figure 2**). Three unknown peaks (**X**, **Y**, and **Z**) were observed as predominant peaks, although they were relatively minor compounds in the reaction of the extract with AAPH. Peaks **1**, **2**, **6**, **X**, **Y**, and **Z** were detected as common reaction products in the reactions of RRA-5, RRA-9, and RRA-11 with AAPH. However, **5** was not detected as a product in the HPLC profile of RRA-5. These results indicate that acylated anthocyanins would have radical scavenging abilities on common sites even if the characteristics of the intramolecular acyl units are different. The occurrence of **1** suggests the nucleophilic attack of water on the 2-position of aglycone, which is in accordance with the results of Tsuda et al. (*20*, *21*). Also, **5** and **6** imply the hydrolysis of the glucose and acyl units, respectively. Garzón and Wrolstad (*31*) reported

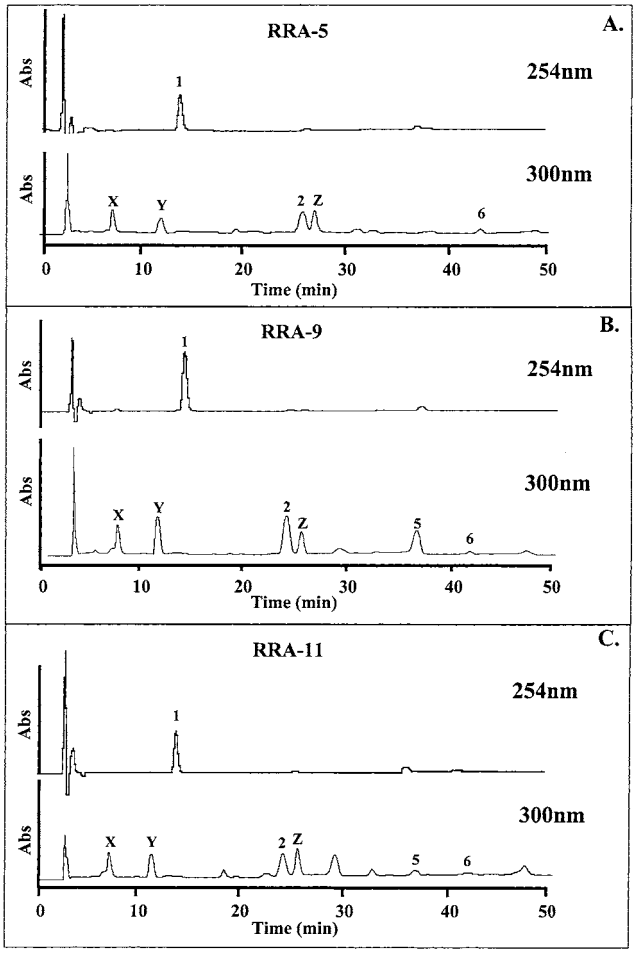


Figure 4. HPLC profiles of the reaction products of acylated anthocyanins with AAPH after 8 days. (A) RRA-5, pelargonidin 3-*O*-[6-*O*-(*E*)-caffeoyl-2-*O*-(6-(*E*)-feruloyl-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside); (B) RRA-9, pelargonidin 3-*O*-[6-*O*-(*E*)-feruloyl-2-*O*-(6-(*E*)-caffeoyl-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside); (C) RRA-11, pelargonidin 3-*O*-[6-*O*-(*E*)- feruoyl-2-*O*-(6-(*E*)-feruloyl-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside).

that changes in pelargonidin acylated with malonic and cinnamic acids during storage resulted in production of malonic acid, and small amounts of pelargonidin 3-sophoroside-5-glucoside and pelargonidin 3-sophoroside. In this experiment, the production of deacylated anthocyanins was not confirmed. The degradation rates of deacylated pelargonidins (pelargonidin and pelargonidin 3-glucoside, callistephin) with AAPH under the same conditions were faster than those of acylated anthocyanins: pelargonidin, a few hours; callistephin, within a day (data not shown). Therefore, to confirm their production, further studies under milder conditions are needed. The identification of unknown compounds (**2**, **X**, **Y**, and **Z**) is currently in progress.

The degradation rates of RRA-5, RRA-9, and RRA-11 and the formation rates of the resulting reaction products were quite different (data not shown). The degradation rates of RRA-5 and RRA-9 were faster than that of RRA-11. These results suggest that the reaction rates of acylated anthocyanins with AAPH radicals are affected by the characteristics of the intramolecular acyl units. The amounts of reaction products from RRA-5 were relatively low compared to that of RRA-9, even though no observable differences were found between the degradation rates. Judging from the degradation and formation behaviors of RRA-5, it may also possess radical scavenging ability on

the other sites. More detailed studies are necessary to clarify the radical scavenging mechanism of acylated anthocyanin.

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