HETEROCYCLES, Vol. 69, 2006, pp. 239 - 251. © The Japan Institute of Heterocyclic Chemistry
Received, 23rd June, 2006, Accepted, 16th August, 2006, Published online, 18th August, 2006. COM-06-S(O)24

ANTHOCYANINS FROM THE RHIZOME OF *RAPHANUS SATIVUS*, AND CHANGE IN THE COMPOSITION DURING MATURATION

Mihoko Mori,[a]† Shuji Nakagawa,[b]† Masayoshi Maeshima,[b] Satoshi Niikura,[c] and Kumi Yoshida[d*]

[a]Graduate School of Human Informatics, Nagoya University, Chikusa, Nagoya 464-8601, Japan; [b]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan; [c]Tohoku Seed Co., Himuro, Utsunomiya, Tochigi 321-3232, Japan; [d]Graduate School of Information Science, Nagoya University, Chikusa, Nagoya 464-8601, Japan. *E-mail address: yoshidak@is.nagoya-u.ac.jp. †The authors contributed equally to this work.

Abstract – Four new acylated anthocyanins with three known pigments were isolated from the rhizome of the Chinese red radish (*Raphanus sativus* cv. Beijing hong xin), and their structures were determined. All of them possessed pelargonidin 3-sophoroside-5-glucoside as a common structure, and each was substituted with one to two aromatic acyl residues, with/without a malonyl residue and with/without a sugar residue. The major aromatic acyl moiety was feruloyl. Most of the pigments were malonylated. The cambium part was richer in polyacylated anthocyanins than the parenchyma part.

INTRODUCTION

The rhizomes of red radish (*Raphanus sativus*) contain a large amount of anthocyanins,[1] and the pigments are now used as a natural food colorant.[1,2] The specific characteristics of radish colorant are high stability and brightness of the red color[3,4] because the major radish anthocyanins are composed of acylated pelargonidins.[1-6] Radish pigments containing aromatic acyl residues (*p*-coumaroyl, caffeoyl and feruloyl) are very stable in a diluted aqueous solution at various pH, and tolerant against heat and light.[2,3,7,8] However, there are many kinds of cultivars in red radishes, and each cultivar has different anthocyanins.[1] For industrial usage, highly anthocyanin-productive cultivars whose pigments exhibit bright color and high stability are in demand. In general, polyacylated anthocyanins bearing two or more aromatic acyl residues in the molecule show higher stability[9] against heat[10] and light[11,12] than monoacylated

anthocyanins. This is due to the intramolecular stacking of aromatic acyl residues to the chromophore preventing the hydration reaction.[9,11-15] Therefore, we studied polyacylated anthocyanins in red radish. Here, we isolated four new anthocyanins from the Chinese red radish, *Raphanus sativus* cv. Beijing hong xin, and determined their structures. We also analyzed the difference in composition of parts of the rhizome tissue and change during maturation.

RESULTS AND DISCUSSION

Extraction and HPLC analysis of anthocyanins in Chinese red radish

The epidermis and cortex of the root of the Chinese red radish (*Raphanus sativus* cv. Beijing hong xin) were colorless, while the inner part was red. When the cultivated rhizome 60 days after sowing, which is almost matured and can be harvested, was cut, a strong red-colored cambium ring was observed at ca. 5 mm from the epidermis. The inner part of the cambium ring was the parenchyma, and this part also contained pigments, although the color was lighter than that of the cambium. Therefore, sliced rhizomes were divided into the two parts, and each was extracted with aq. MeCN containing trifluoroacetic acid (TFA) separately. The extracts were analyzed using photo-diode array detection ODS-HPLC. As shown in Figure 1, the red radish contained many anthocyanins, and the anthocyanin compositions of cambium and parenchyma were different from each other. All the peaks showed λvismax around 510 nm, indicating that all of them had a pelargonidin nucleus. The ratio of the absorbance at λuvmax to λvismax suggests the number of aromatic acyl moieties.[13,15] The spectrum of each peak indicated that **1**-**3** might have one cinnamoyl derivative-residue, while **4**-**7** might have two.

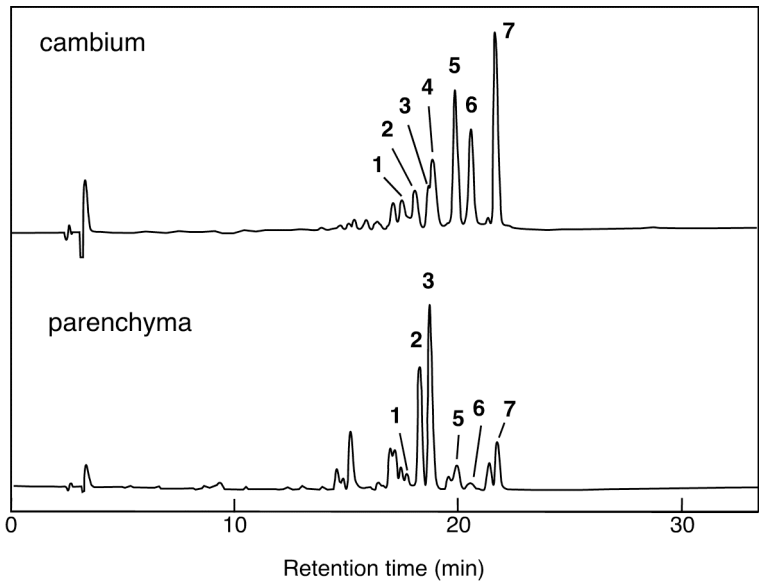


Figure 1. The difference in anthocyanin composition between the extract of the cambium part (upper) and the parenchyma part (lower) of a red rhizome of *Raphanus sativus* cv. Beijing hong xin, analyzed by HPLC (detection: 530 nm).

Isolation of anthocyanins in Chinese red radish

Because the composition of each part of rhizome was different, the sliced root tissue was separated into the cambium and parenchyma part for large-scale extraction. For extraction and purification, we used acidic MeCN instead of acidic methanol to prevent demalonylation. To the freshly frozen parenchyma part (700 g) was added 4 L of 3% TFA-50% aq. MeCN, and the extract was treated with Amberlite XAD-7 column chromatography. The column was eluted with aq. MeCN containing TFA to give a crude pigment fraction. The fraction was purified with a preparative ODS HPLC (gradient elution from aq. TFA to 50% aq. MeCN containing TFA) repeatedly according to our general procedure[12,15-18] to give **1** (3.8 mg), **2** (4.8 mg) and **3** (3.5 mg) as dark red TFA salts. From the cambium parts (42 g), the anthocyanins were extracted and purified using the same procedure to give **4** (1.3 mg), **5** (2.6 mg), **6** (1.9 mg), and **7** (1.8 mg).

Structure determination of radish anthocyanins

The structure of each anthocyanin was determined by ESI-TOF MS and various 1D and 2D NMR experiments (Table 1-3). The ESI-TOF MS spectra of **1**-**7** showed the same fragment ion peak at *m*/*z* = 271, indicating that all the pigments had a pelargonidin nucleus. **1**, **2**, **3**, **6** and **7** showed a fragment ion peak of 86 mass units smaller than the molecular ion peak, suggesting that they had a malonyl residue in the molecule. Combined with the estimated number of acyl moieties, the components of each pigment were determined (Table 1).

Table 1. Molecular weight of each anthocyanins and the estimated numbers of malonyl, aromatic acyl, and sugar residues.

	m/*z* (M^+)	malonyl residue	aromatic acyl residue	sugar residue
1	1181	1	1	4
2	989	1	1	3
3	1019	1	1	3
4	1271	0	2	4
5	1109	0	2	3
6	1357	1	2	4
7	1195	1	2	3

First, we deduced the structure of the largest-molecular-weight anthocyanin (**6**), and its demalonyl derivative (**4**). From the MS data, it was indicated that **6** contained two aromatic acyl residues, four sugars, and one malonyl moiety with a pelargonidin nucleus (Table 1). The molecular weight of **4** (*m*/*z* = 1271) and the similarity between the ^{1}H NMR spectra of **4** and **6** strongly suggested that **4** was a demalonylated

compound from **6** (Table 1). To confirm this, **6** was treated with HCl-methanol, and the reaction was monitored by HPLC. With 12 hrs' reaction, a new peak whose retention time was longer than that of **6** appeared, and after that, one more new peak appeared that was identical to **4**. These results were very similar in behavior to malonylated anthocyanins.[15,17,18] Therefore, **4** was confirmed to have a demalonate from **6**. Thus, we analyzed the NMR spectra of **4** before the structural determination of **6**. ^{1}H NMR of **4** showed the existence of two (*E)*-feruloyl residues and four sugars with a pelargonidin nucleus (Table 2). By 1D- and 2D TOCSY experiments,[12,13,15,19] the signals of the sugar residues of **4** assigned all of the sugars to *β*-glucopyranosides (Table 2). The methylene protons at the 6-position of the sugar residues (glc-1 and glc-2) showed a downfield shift, indicating that 6-OH of both glucosides were acylated by ferulic acid. To determine the linkage of glc-4, HMQC, HMBC, and NOESY experiments were conducted. By the NOESY experiment, NOEs between the anomeric proton of glc-4 and the protons at 3 and 4 of glc-3 were observed, suggesting that glc-4 was connected to either 3 or 4-OH of glc-3 (Figure 2). However, further assignment was impossible because the signals of glc-3 and glc-4 were overlapped. Therefore, further NMR experiments of **4** in 5% TFA-*d*-DMSO-d_6 were carried out. By 1D TOCSY and HMQC, the ^{13}C signal at 80.1 ppm was assigned to be 4-position of glc-3. The HMBC correlation was observed between the ^{1}H signal of 1-position of glc-4 and this ^{13}C signal at 80.1 ppm. These data indicated that the glc-4 was linked to 4-OH of glc-3. The attached positions of two feruloyl residues were confirmed to be 6-OH of glc-1 and glc-2 from the correlation of HMBC. Thus, the structure of **4** was deduced to be 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-(6-*O*-(*E*)-feruloyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl)-5-*O*-(4-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)pelargonidin.

The methylene signals of the 6-position of glc-3 in **6** were observed at 4.44 and 4.60 ppm, which was shifted downfield compared with those of **4**. This indicated that a malonyl residue was connected at 6-OH of glc-3. Therefore, the structure of **6** was determined to be 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-(6-*O*-(*E*)-feruloyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl)-5-*O*-(4-*O*-*β*-D-glucopyranosyl-6-*O*-malonyl-*β*-D-glucopyranosyl)pelargonidin (Scheme 1).

Using the same NMR experiments as described above, the structure of **7** was deduced to be 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-(6-*O*-(*E*)-feruloyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)pelargonidin. The difference in 86 mass units between **7** and **5**, and the similarity between ^{1}H NMR of **7** and **5** indicated that **5** was a demalonylated derivative of **7**. This was confirmed by HCl-MeOH treatment of **7** to give **5**.[15,17,18] Therefore, pigment **5** was identified as 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-(6-*O*-(*E*)-feruloyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl)-5-*O*-*β*-D-glucopyranosylpelargonidin.[4]

The MS data and ^{1}H NMR of **1** showed the existence of one feruloyl, one malonyl and four glucosyl

Table 2. Assignment of ^{1}H NMR spectra of anthocyanins (**1**-**7**) (5%TFA*d*-CD_3OD, 20˚C) with *J* values.

position		**1**			**2**			**3**			**4**		
		(ppm)		*J* (Hz)	(ppm)		*J* (Hz)	(ppm)		*J* (Hz)	(ppm)		*J* (Hz)
4		8.97	s		8.97	s		8.99	s		8.92	s	
6		6.95	d	1.5	6.97	d	2.0	6.98	d	2.0	6.82	d	2.0
8		6.86	d	1.5	6.98	d	2.0	6.95	d	2.0	6.65	d	2.0
2', 6'		8.57	d	9.0	8.57	d	9.0	8.58	d	9.0	8.46	d	9.0
3', 5'		7.08	d	9.0	7.08	d	9.0	7.08	d	9.0	6.97	d	9.0
F1	2	6.96	d	1.5				6.87	d	2.0	6.90	d	2.0
	5	6.73	d	8.0				6.73	d	8.0	6.78	d	8.5
	6	6.83	dd	8.0, 1.5				6.83	dd	8.0, 2.0	6.82	dd	8.5, 2.0
	α	6.17	d	16.0				6.18	d	16.0	5.95	d	16.0
	β	7.33	d	16.0				7.35	d	16.0	7.23	d	16.0
	OCH_3	3.80	s					3.79	s		3.84	s	
F2	2										6.66	d	2.0
	5										6.62	d	8.5
	6										6.57	dd	8.5, 2.0
	α										5.82	d	16.0
	β										7.01	d	16.0
	OCH_3										3.71	s	
pC	2, 6				7.20	d	8.5						
	3, 5				6.71	d	8.5						
	α				6.18	d	16.0						
	β				7.33	d	16.0						
glc-1	1	5.61	d	7.5	5.60	d	7.5	5.59	d	7.5	5.29	d	7.5
	2	4.11	dd	9.5, 7.5	4.10	dd	9.0, 7.5	4.11	dd	9.0, 7.5	3.93	dd	9.5, 7.5
	3	3.85	t	9.5	3.83	t	9.0	3.83	t	9.0	3.74	t	9.5
	4	3.63	t	9.5	3.60	t	9.0	3.64	t	9.0	3.59	t	9.5
	5	3.95	ddd	9.5, 6.0, 2.0	3.93	ddd	9.0, 8.0, 2.0	3.94	ddd	9.0, 7.5, 2.5	3.63	ddd	9.5, 6.5, 2.5
	6a	4.39	dd	11.5, 6.0	4.43	dd	12.0, 8.0	4.37	dd	12.0, 7.5	4.34	dd	12.0, 6.5
	6b	4.58	dd	11.5, 2.0	4.52	dd	12.0, 2.0	4.59	dd	12.0, 2.5	4.41	dd	12.0, 2.5
glc-2	1	4.76	d	7.5	4.76	d	7.5	4.76	d	7.5	4.75	d	7.5
	2	3.32	dd	9.5, 7.5	3.29	dd	8.5, 7.5	3.21	dd	9.0, 7.5	3.40	m	
	3	3.24	t	9.5	3.22	t	8.5	3.31	t	9.0	3.44	m	
	4	3.22	t	9.5	3.21	t	8.5	3.23	t	9.0	3.44	m	
	5	3.01	ddd	9.5, 6.5, 2.5	2.99	ddd	8.5, 5.5, 2.0	3.01	ddd	9.0, 6.0, 3.0	3.53	m	
	6a	3.47	dd	12.0, 6.5	3.46	dd	12.0, 5.5	3.46	dd	12.5, 6.0	4.27	dd	12.0, 5.5
	6b	3.56	dd	12.0, 2.5	3.54	dd	12.0, 2.0	3.56	dd	12.5, 3.0	4.31	dd	12.0, 2.0
glc-3	1	5.23	d	7.5	5.20	d	7.5	5.18	d	7.5	5.09	d	7.5
	2	3.85	dd	9.5, 7.5	3.78	dd	9.0, 7.5	3.77	dd	9.0, 7.5	3.75	dd	9.0, 7.5
	3	3.74	t	9.5	3.57	t	9.0	3.55	t	9.0	3.70	t	9.0
	4	3.67	t	9.5	3.46	t	9.0	3.43	t	9.0	3.68	t	9.0
	5	3.99	ddd	9.5, 6.0, 1.5	3.81	ddd	9.0, 6.0, 2.0	3.76	ddd	9.0, 6.0, 2.0	3.63	ddd	9.0, 4.0, 2.0
	6a	4.41	dd	11.5, 6.0	4.25	dd	12.0, 6.0	4.12	dd	12.0, 6.0	3.91	dd	12.0, 4.0
	6b	4.60	dd	11.5, 1.5	4.54	dd	12.0, 2.0	4.54	dd	12.0, 2.0	3.96	dd	12.0, 2.0
glc-4	1	4.39	d	7.5							4.46	d	8.0
	2	3.25	dd	9.0, 7.5							3.28	dd	9.5, 8.0
	3	3.41	t	9.0							3.41	m	
	4	3.33	t	9.0							3.34	m	
	5	3.47	ddd	9.0, 5.5, 2.5							3.41	m	
	6a	3.69	dd	12.0, 5.5							3.70	m	
	6b	3.93	dd	12.0, 2.5							3.92	m	

Table 2. (continued)

position		**5**			**6**			**7**		
		(ppm)		*J* (Hz)	(ppm)		*J* (Hz)	(ppm)		*J* (Hz)
4		8.91	s		8.93	s		8.94	s	
6		6.83	d	2.5	6.82	d	2.0	6.83	d	2.0
8		6.62	d	2.5	6.69	d	2.0	6.67	d	2.0
2', 6'		8.46	d	9.0	8.47	d	9.0	8.47	d	9.0
3', 5'		6.96	d	9.0	6.99	d	9.0	6.98	d	9.0
F1	2	6.89	d	2.0	6.88	d	2.0	6.88	d	2.0
	5	6.77	d	8.0	6.76	d	8.0	6.76	d	8.0
	6	6.82	dd	8.0, 2.0	6.82	dd	8.0, 2.0	6.81	dd	8.0, 2.0
	α	5.94	d	16.0	5.99	d	16.0	5.98	d	16.0
	β	7.23	d	16.0	7.24	d	16.0	7.25	d	16.0
	OCH_3	3.84	s		3.84	s		3.82	s	
F2	2	6.65	d	2.0	6.69	d	2.0	6.68	d	2.0
	5	6.61	d	8.5	6.65	d	8.0	6.63	d	8.0
	6	6.56	d	8.5, 2.0	6.61	dd	8.0, 2.0	6.60	dd	8.0, 2.0
	α	5.81	d	16.0	5.83	d	16.0	5.82	d	16.0
	β	7.02	d	16.0	7.02	d	16.0	7.02	d	16.0
	OCH_3	3.71	s		3.73	s		3.72	s	
pC	2, 6									
	3, 5									
	α									
	β									
glc-1	1	5.24	d	7.5	5.36	d	7.5	5.30	d	7.5
	2	3.92	dd	9.0, 7.5	3.95	dd	9.0, 7.5	3.94	dd	9.0, 7.5
	3	3.74	t	9.0	3.76	t	9.0	3.75	t	9.0
	4	3.59	t	9.0	3.58	t	9.0	3.57	t	9.0
	5	3.65	ddd	9.0, 7.0, 2.5	3.74	ddd	9.0, 7.0, 2.0	3.70	ddd	9.0, 6.0, 2.0
	6a	4.35	dd	12.0, 7.0	4.33	dd	12.0, 7.0	4.32	dd	12.0, 6.0
	6b	4.42	dd	12.0, 2.5	4.42	dd	12.0, 2.0	4.42	dd	12.0, 2.0
glc-2	1	4.75	d	7.5	4.76	d	7.5	4.77	d	7.5
	2	3.42	dd	9.0, 7.5	3.44	dd	9.0, 7.5	3.45	dd	9.0, 7.5
	3	3.47	t	9.0	3.44	m		3.44	m	
	4	3.45	t	9.0	3.44	m		3.45	m	
	5	3.54	ddd	9.0, 5.5, 2.5	3.46	m		3.48	m	
	6a	4.29	dd	12.0, 5.5	4.22	dd	12.0, 5.0	4.24	dd	12.0, 5.0
	6b	4.33	dd	12.0, 2.5	4.29	dd	12.0, 2.0	4.33	dd	12.0, 1.5
glc-3	1	5.10	d	7.5	5.05	d	7.5	5.05	d	7.5
	2	3.70	dd	9.0, 7.5	3.76	dd	9.0, 7.5	3.70	dd	9.0, 7.5
	3	3.55	t	9.0	3.68	t	9.0	3.53	t	9.0
	4	3.45	t	9.0	3.66	t	9.0	3.45	t	9.0
	5	3.51	ddd	9.0, 7.0, 2.5	3.84	ddd	9.0, 6.0, 2.0	3.67	ddd	9.0, 7.0, 2.0
	6a	3.73	dd	12.0, 7.0	4.44	dd	12.0, 6.0	4.24	dd	12.0, 7.0
	6b	3.95	dd	12.0, 2.5	4.60	dd	12.0, 2.0	4.53	dd	12.0, 2.0
glc-4	1				4.40	d	7.5			
	2				3.25	dd	9.0, 7.5			
	3				3.40	t	9.0			
	4				3.32	t	9.0			
	5				3.42	ddd	9.0, 6.0, 2.0			
	6a				3.70	dd	12.0, 6.0			
	6b				3.91	dd	12.0, 2.0			

Table 3. Assignment of ^{13}C NMR spectra of anthocyanins (**1**, **4**, **6** and **7**) (5%TFA*d*-CD_3OD, 20˚C).

position		**1**	**4**	**6**	**7**
2		164.1	165.7	165.6	165.6
3		145.8	144.9	145.3	145.2
4		134.4	140.4	139.5	139.9
5		155.2	156.9	156.7	156.8
6		104.7	105.8	106.3	106.1
7		169.1	170.3	169.9	170.1
8		96.1	97.6	97.6	97.5
9		155.2	157.2	157.2	157.2
10		111.9	113.3	113.4	113.3
1’		119.3	120.3	120.4	120.3
2’,6’		135.0	136.3	136.3	136.3
3’,5’		116.9	118.1	118.1	118.1
4’		164.1	167.3	167.4	167.3
acyl-1	1	125.9	127.2	127.2	127.2
	2	110.9	111.8	111.9	111.8
	3	147.8	149.3	149.3	149.2
	4	147.8	150.7	150.8	150.7
	5	115.1	116.5	116.5	116.5
	6	122.6	124.0	124.0	124.0
	α	112.0	114.7	114.8	114.8
	β	145.8	147.0	147.0	147.0
	C=O	167.6	168.7	168.6	168.7
	OCH_3	55.2	56.5	56.5	56.5
acyl-2	1		126.9	126.9	126.0
	2		111.0	111.1	111.1
	3		149.2	149.2	149.1
	4		150.6	150.7	150.6
	5		116.4	116.4	116.4
	6		123.9	124.0	124.0
	α		114.7	114.7	114.6
	β		146.4	146.4	146.5
	C=O		168.6	168.5	168.5
	OCH_3		56.3	56.3	56.3
glc-1	1	103.8	103.4	103.4	103.5
glc-2	1	103.4	106.2	106.1	106.0
glc-3	1	106.4	102.3	102.3	102.5
glc-4	1	103.6	104.6	104.7	
mal	C=O	167.0		168.0	170.4

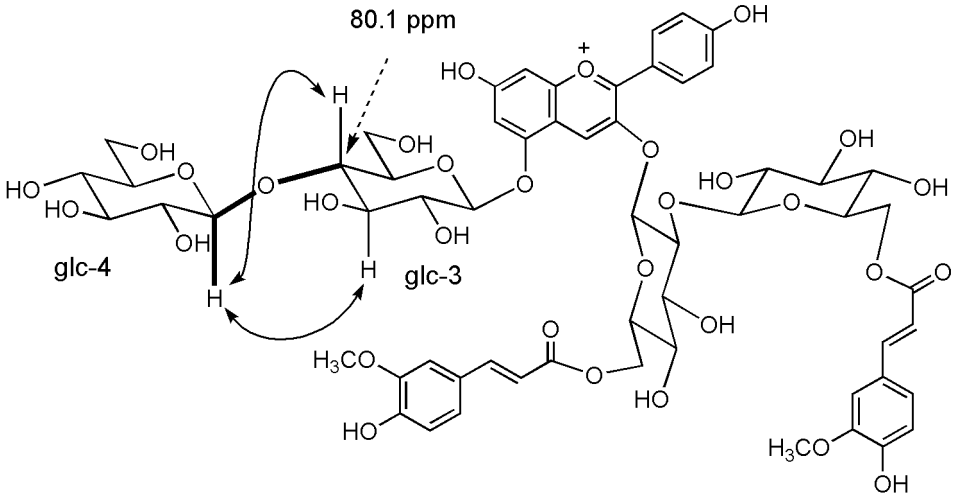
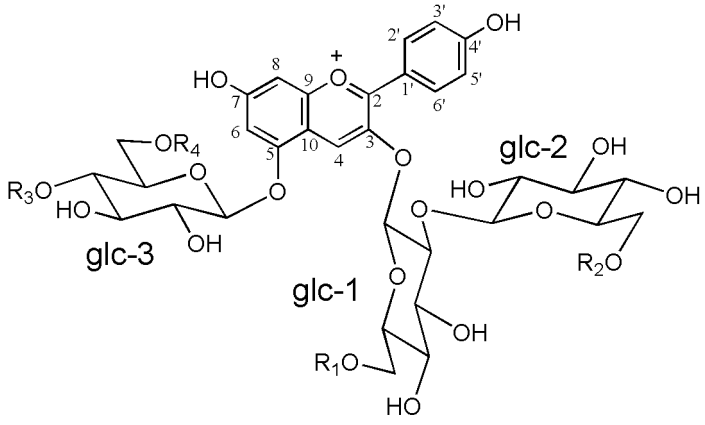


Figure 2. NOE and HMBC correlations of **4**. NOE correlations are indicated with double-pointed arrows, and HMBC correlations are drawn with thicker lines.



	R_1 (acyl-1)	R_2 (acyl-2)	R_3	R_4
1	feruloyl (F1)	H	glucose (glc-4)	malonyl
2	*p*-coumaroyl (pC)	H	H	malonyl
3	feruloyl (F1)	H	H	malonyl
4	feruloyl (F1)	feruloyl (F2)	glucose (glc-4)	H
5	feruloyl (F1)	feruloyl (F2)	H	H
6	feruloyl (F1)	feruloyl (F2)	glucose (glc-4)	malonyl
7	feruloyl (F1)	feruloyl (F2)	H	malonyl

Scheme 1. Structures of anthocyanins in rhizome of red radish, *Raphanus sativus* cv. Beijing hong xin.

residues with a pelargonidin nucleus. The signals of the sugar region were similar to those of **4** and **6**. The downfield shift of the methylene protons of glc-1 and glc-3 indicated that the 6-position of both glc-1 and glc-3 were acylated. Using the same NMR experiments, the structure of **1** was determined to be 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)-5-*O*-(4-*O*-*β*-D-glucopyranosyl-6-*O*-malonyl-*β*-D-glucopyranosyl)pelargonidin (Scheme 1).

^{1}H NMR of **2** and **3** showed the existence of one (*E*)-*p*-coumaroyl or one (*E*)-feruloyl residue, respectively (Table 2). The other signals in the ^{1}H NMR of **2** and **3** were very similar. Using the same procedure described above, the structure of **2** was identified to be 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl-2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)pelargonidin[6] and that of **3** was identified to be 3-*O*-(6-*O*-(*E*)-feruloyl-2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-*β*-D-glucopyranosyl)pelargonidin[6] (Scheme 1).

Anthocyanins (**1**, **4**, **6** and **7**) are newly isolated pigments. In this study, most of the pigments were malonylated, although in previous reports of red radish anthocyanins, the existence of malonyl residue was little or none. In previous studies,[4,6,7] the radish pigments were extracted with an acidic methanol solution or aq. sulfate. Malonyl ester must be easily hydrolyzed under these severe conditions, so previously reported demalonylated anthocyanins may have been degradation products during extraction and purification. Malonyl residues may play a role, such as biosynthesis and/or transportation, in radish tissue.

Change in anthocyanin composition of red radish during maturation

After 20 days from sowing, the radish roots became ca. 10 mm in diameter. At that time, the cambium and pericycle parts showed strong red color, but only the center of the parenchyma part showed red color while the other part was colorless. However, during maturation, the color of the cambium faded and that of the parenchyma became a stronger red. The diameter of the 60-day-old rhizome was ca. 60 mm, and at that time, the parenchyma showed red entirely. To clarify the change in anthocyanin composition during rhizome maturation, 20-, 40- and 60-day-old roots were harvested, and each cambium and parenchyma part was extracted and analyzed by HPLC (Figure 3).

In a 20-day-old plant, the major pigments of the cambium part were monoferuloyl anthocyanin (**3**), and diferuloylanthocyanin (**7**), while in the parenchyma part, **3** was dominant. During maturation, the composition of each part changed a little; in the cambium, the content of **2** increased slightly, while in the parenchyma, the increase in **2** was remarkable, and the contents of the other polyacylated anthocyanins (**5**), (**6**) and (**7**), increased slightly. In both parts, **4** was a minor pigment whose peak was very small. The total anthocyanin content per fresh weight was gradually decreased along with rhizome maturation, although the total amount of pigments in a rhizome became very high.

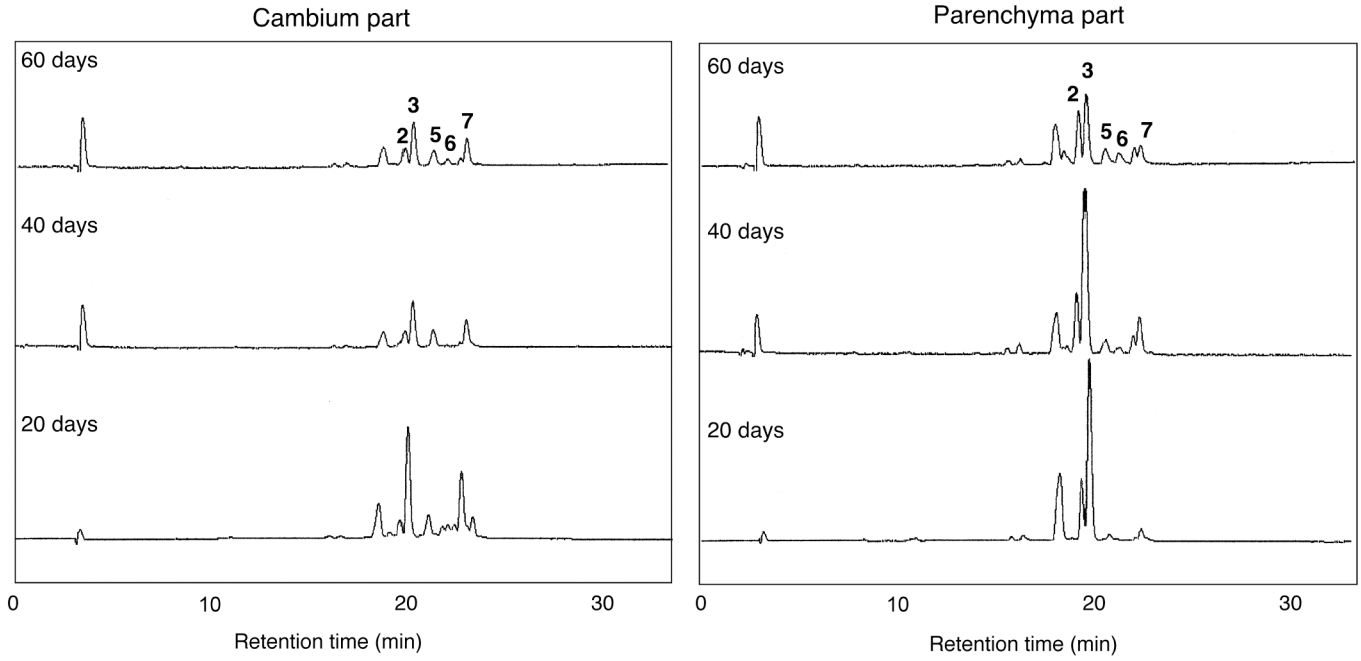


Figure 3. Change in anthocyanin composition of the red rhizome of *Raphanus sativus* cv. Beijing hong xin, during maturation. The extracts of the cambium part (left) and the parenchyma part (right) and at 20, 40 and 60 days' old were analyzed by HPLC (detection: 530 nm).

In conclusion, red radish, *Raphanus sativus* cv. Beijing hong xin, is a rich source of acylated anthocyanins. The major substituted aromatic acid was ferulic acid, and more than 90% of the pigment contained malonyl residue. The composition of anthocyanins in rhizomes is different the between cambium and the parenchyma part. In the cambium part, the content of polyacylated anthocyanins was higher than that in the parenchyma. There must be some difference in the biosynthetic process, and malonylation might play a role in transportation.

EXPERIMENTAL

General

UV-VIS spectra and reflection spectra were recorded on a JASCO V-560 spectrometer. NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) and a JNM-A600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz) in a 5-mm ϕ tube at variable temperature using 5-10% TFA*d*-CD_3OD as a solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD resonance as a standard. ESI-TOF MS data were recorded on a PE Biosystems QSTAR and analyzed with PE SCIEX

Analyst QA software. Analytical and preparative HPLC were conducted according to our procedure[11-13,15-18] using an ODS-column.

Plant materials

To isolate the anthocyanins of red radish, *Raphanus sativus* cv. Beijing hong xin was cultivated at the Farm of Tohoku Seed Co. for two months and harvested. The fresh rhizomes were divided into the parenchyma and cambium parts, and then both pieces were cut into ca. 3-mm-thick slices and stored at -30°C until extraction. For analysis of change in the anthocyanin content, the seeds distributed from Tohoku Seed Co. were cultivated at Nagoya University Farm from autumn 2003 to spring 2004. The roots were harvested over time and cut into the parenchyma and cambium parts, and then stored at -30°C until use.

Extraction and HPLC analysis of anthocyanins from red radish

To a small piece of cut radish was added 50% aq. MeCN solution containing 3% TFA, and it was then allowed to stand at rt for 18 h. The extract was analyzed with HPLC using an ODS-column (Develosil ODS-HG-5 2.0 mm ϕ X 250 mm, Nomura Chemical) eluted with thirty-minute linear gradient elution from 10% to 30% aq. MeCN solution containing 0.5% TFA at 40°C.[15,18]

Isolation of anthocyanins (1-3) from parenchyma part of red radish

The sliced pieces of the red-colored parenchyma part (700 g) were extracted with 4 L of 50% aq. MeCN solution containing 3.0% TFA. The extract was evaporated under reduced pressure to 1 L, and the condensed extract was purified with an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 0.3% TFA-H_2O to 35% aq. MeCN solution containing 0.3% TFA. The 25%-30% aq. MeCN fraction was evaporated to give a crude mixture of anthocyanins (**1**-**3**) (1.3 g). By further purification with a preparative ODS-HPLC (Develosil ODS-HG-5 20 mm ϕ X 250 mm, Nomura Chemical) eluted with 18% aq. MeCN solution containing 0.5% TFA, **1** (3.8 mg), **2** (4.8 mg) and **3** (3.5 mg) were obtained as red amorphous TFA salts.

Anthocyanin (**1**). UV/VIS (0.1%HCl-MeOH) λmax nm (ε): 509 (24,600), 329 (15,800), 286 (17,500).

Isolation of anthocyanins (4-7) from cambium part of red radish

The sliced pieces of the red-colored cambium part (42 g) were extracted with 0.56 L of 50% aq. MeCN solution containing 2.0% TFA. The extract was evaporated under reduced pressure to 70 mL, and the condensed extract was purified with an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 0.5% TFA-H_2O to 40% aq. MeCN solution containing 0.5% TFA. The

30% aq. MeCN fraction was evaporated to give a crude mixture of anthocyanins (**4**-**7**) (56 mg). The crude anthocyanins were purified using a preparative ODS-HPLC to give **4** (1.3 mg), **5** (2.6 mg), **6** (1.9 mg) and **7** (1.8 mg) as red amorphous TFA salts.

Anthocyanin (**4**). UV/VIS (0.1%HCl-MeOH) λmax nm (ε): 509 (24,600), 328 (26,500), 287 (22,500).

Anthocyanin (**6**). UV/VIS (0.1%HCl-MeOH) λmax nm (ε): 509 (23,100), 328 (26,000), 288 (22,100).

Anthocyanin (**7**). UV/VIS (0.1%HCl-MeOH) λmax nm (ε): 508 (26,700), 328 (27,700), 287 (23,700).

ACKNOWLEDGEMENTS

We are grateful to Ms. N. Yoshino of Nagoya University for the cultivation of plant materials. This work was financially supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan ((B) No. 16370021 and No. 16380068, The 21st Century COE Program No. 14COEB01-00 and No. 14COEA02, and Creative Scientific Research No.16GS0206).

REFERENCES (AND NOTES)

1. G. Mazza and E. Miniati, 'Anthocyanins in Fruits, Vegetables, and Grains,' CRC Press, Boca Raton, 1993, pp. 288-290.
2. M. M. Giusti and R. E. Wrolstad, *Biochem. Eng. J.*, 2003, **14**, 217.
3. C. Malien-Aubert, O. Dangles, and M. J. Amiot, *J. Agric. Food Chem.*, 2001, **49**, 170.
4. T. Otsuki, H. Matsufuji, M. Takeda, M. Toyoda, and Y. Goda, *Phytochemistry*, 2002, **60**, 79.
5. T. Shimizu, T. Ichi, H. Iwabuchi, Y. Kato, and Y. Goda, *J. Jpn. Soc. Food Sci. Tech.*, 1996, **3**, 5.
6. M. M. Giusti, H. Ghanadan, and R. E. Wrolstad, *J. Agric. Food Chem.*, 1998, **46**, 4858.
7. M. M. Giusti and R. E. Wrolstad, *J. Food Sci.,* 1996, **61**, 322.
8. L. E. Rodriguez-Saona, M. M. Giusti, and R. E. Wrolstad, *J. Food Sci.*, 1999, **64**, 451.
9. T. Goto and T. Kondo, *Angew. Chem. Int. Ed. Engl.*, 1991, **30**, 17.
10. G. Mazza and E. Miniati, 'Anthocyanins in Fruits, Vegetables, and Grains,' CRC Press, Boca Raton, 1993, pp. 10-20.
11. K. Yoshida, R. Okuno, K. Kameda, M. Mori, and T. Kondo, *Biochem. Engineer. J.*, 2003, **14**, 163.
12. K. Yoshida, M. Mori, M. Kawachi, R. Okuno, K. Kameda, and T. Kondo, *Tetrahedron Lett.*, 2003, **44**, 7875.
13. K. Yoshida, T. Kondo, and T. Goto, *Tetrahedron*, 1992, **48**, 4313.
14. O. M. Anderson and M. Jordheim, 'Flavonoids Chemistry, Biochemistry and Applications,' ed. by O. M. Anderson and K. R. Markham, CRC Press: Boca Raton, 2006, pp. 471-551.
15. M. Mori, T. Kondo, K. Toki, and K. Yoshida, *Phytochemistry*, 2006, **67**, 622.
16. K. Yoshida, Y. Sato, R. Okuno, K. Kameda, M. Isobe, and T. Kondo, *Biosci. Biotech. Biochem.*,

1996, **60**, 589.

17. K. Yoshida, K. Kameda, and T. Kondo, '*Perilla*,' ed. by Y. He-ci, K. Kosuna, and M. Haga, Harwood Academic Publishers, Amsterdam, 1997, pp. 149-169.
18. K. Yoshida, S. Kitahara, D. Ito, and T. Kondo, *Phytochemistry*, 2006, **67**, 992.
19. T. Kondo, M. Ueda, and T. Goto, *Tetrahedron*, 1990, **46**, 4749.