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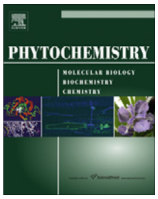

Major anthocyanins from purple asparagus (*Asparagus officinalis*)

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ABSTRACT

Two major anthocyanins (A1 and A2) were isolated from peels of the spears of *Asparagus officinalis* cv. Purple Passion. They were purified by column, paper and high-performance liquid chromatographic separations, and their structures were elucidated by high-resolution Fourier transform ion cyclotron resonance mass spectrometry (HR-FT-ICR MS), ^{1}H, ^{13}C and two-dimensional NMR spectroscopic analyses and either acid or alkaline hydrolysis, respectively. A1 was identified as cyanidin 3-[3″-(*O*-β-D-glucopyranosyl)-6″-(*O*-α-L-rhamnopyranosyl)-*O*-β-D-glucopyranoside], whereas A2 was cyanidin 3-rutinoside, which is widely distributed in higher plants. Oxygen radical absorbance capacity (ORAC) assays proved their high antioxidant activities.

1. Introduction

Asparagus officinalis is known to be rich in functional components such as rutin (quercetin 3-rutinoside) (Griffith et al., 1955), saponin (Chin et al., 2002) and glutathione (Demirkol et al., 2004). Green or etiolated white spears are harvested in this plant. The green spears have a small amount of anthocyanin, and the spears often take on a red tinge. Recently, asparagus with reddish purple spears has attracted consumers. Purple asparagus is expected to have a higher antioxidant activity than the green or white varieties, because of the increased levels of its abundant anthocyanins. In fact, Maeda et al. (2005) suggested that spears of purple asparagus have high antioxidant activities. In order to evaluate the usefulness of purple asparagus, it is necessary to clarify the structures of its anthocyanins and their functions.

There are several reports on anthocyanin accumulation in green asparagus spears. The oldest description is in the review by Robinson and Robinson (1934) indicating that the anthocyanin in asparagus was a cyanidin (Cy) diglycoside. Wann and Thompson (1965) isolated two anthocyanins from asparagus spears and identified them as Cy 3-glucoside and Cy 3,5-diglucoside by PC. They also suggested that they were acylated by carboxylic acid. Francis (1967) identified four anthocyanins: Cy 3-glucosylrhamnosylglucoside, peonidin (Pn) 3-glucosylrhamnosylglucoside, Cy 3-rhamnosylglucoside and Pn 3-rhamnosylglucoside, and found that none were acylated. There is no report on anthocyanins in other *Asparagus* species, the only one genus in the Asparagaceae.

In this paper, we report the structural elucidation and the antioxidant activities of anthocyanins from the purple spears of *A. officinalis* cv. Purple Passion.

2. Results and discussion

Two major anthocyanin peaks (A1 and A2) were observed in the 10% HCO_2H extract by HPLC with monitoring at 530 nm. The proportions were 33.5% (A1) and 47.9% (A2) of the total absorbance of the anthocyanin peaks.

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Acid hydrolysis of both A1 and A2 produced cyanidin, glucose and rhamnose. The HPLC retention times of A1 and A2 did not change after alkaline hydrolysis (data not shown), indicating that they had no acyl moieties.

2.1. Compound **1** (A1)

A1 showed three absorption maxima at 530, 332 and 282 nm in the UV–vis region in 0.1% HCl–MeOH. Since two intermediates (Cy 3-glucoside and Cy 3-rutinoside) were detected by moderate acid hydrolysis, A1 was suggested to be a cyanidin triglycoside containing glucose and rhamnose moieties. HR-FT-ICR MS gave a molecular ion peak at m/z 757.21880 $[M]^+$ indicative of molecular formula $C_{33}H_{41}O_{20}$ (calculated: 757.21857; error: 0.3 ppm). Fragment ion peaks were detected at m/z 595.1659 $[M–Glc(162.0527)]^+$, 449.1077 $[M–Glc–Rha(146.0582)]^+$ and 287.0552 $[M–2Glc(162.0526)–Rha]^+$ corresponding to flavylium ions of cyanidin rutinoside, cyanidin glucoside and cyanidin, respectively. The MALDI-TOF MS data was similar to that of the HR-FT-ICR MS. Full assignment data for the ^1H NMR and ^{13}C NMR spectra using $^1H\{^{13}C\}$ HSQC, $^1H\{^{13}C\}$ HMBC, TOCSY, DQF-COSY, NOESY and ROESY are summarized in Table 1.

^1H NMR and ^{13}C NMR spectroscopic data indicated the presence of the (two) glucosyl and (one) rhamnosyl residues. In analyzing the $^1H\{^{13}C\}$ HMBC spectrum, H-1 of Glc-1 (δ 5.27) had a cross-peak to the C-3 carbon (δ 145.47) on the Cy A-ring, and NOESY between H-1 of Glc-1 and H-4 of the Cy A-ring. This suggested that one Glc was attached to the C-3 carbon of the Cy A-ring. H-1 in Glc-2 (δ 4.56) and Rha (δ 4.64) showed $^1H\{^{13}C\}$ HMBC cross-peaks to the C-3 (δ 83.35) and C-6 (δ 67.85) carbons in Glc-1, respectively. Both carbon signals showed downfield shift changes. NOESY cross-peaks between H-3 of Glc-1 and H-1 of Glc-2, and between H-6 of Glc-1 and H-1 of Rha, were observed. These data indicated that Glc-1 was glycosylated by Glc-2 at the C-3 position and by Rha at the C-6 position. From these results, A1 was identified as cyanidin 3-[3″-(*O*-β-D-glucopyranosyl)-6″-(*O*-α-L-rhamnopyranosyl)-*O*-β-D-glucopyranoside] (**1**) (Fig. 1).

Table 1
^1H and ^{13}C NMR spectroscopic data of A1 and A2

	A1			A2		
	^1H (δ)	*J* (Hz)	^{13}C (δ)	^1H (δ)	*J* (Hz)	^{13}C (δ)
Cyanidin						
2			164.42			164.40
3			145.47			145.82
4	8.93	*s*	136.58	8.93	*s*	136.38
5			159.15			159.24
6	6.69	*d* (1)	103.58	6.68	*d* (1)	103.65
7			170.55			170.69
8	6.91	*d* (1)	95.33	6.89	*d* (1)	95.39
9			157.70			157.87
10			113.26			113.43
1′			121.23			121.40
2′	8.03	*d* (2.2)	118.47	8.03	*d* (2.2)	118.48
3′			147.46			117.63
4′			155.87			156.07
5′	7.03	*d* (8.7)	117.49	7.02	*d* (8.7)	117.43
6′	8.26	*dd* (2.2,8.7)	128.44	8.26	*dd* (8.7,2.2)	128.58
Glucose-1						
1″	5.27	*d* (7.7)	103.48	5.28	*d* (7.7)	103.68
2″	3.66	*dd* (9,8)	74.73	3.67	*dd* (9,8)	74.85
3″	3.54	*t* (9)	83.35	3.56	*t* (9)	78.19
4″	3.42	*t* (9)	71.19	3.42	*t* (9)	71.40
5″	3.70	*m*	77.36	3.72	*ddd* (9,7,1)	77.61
6″	3.62	*dd* (11,6)	67.85	3.60	*dd* (11,7)	67.98
	4.03	*dd* (11,1)		4.07	*dd* (11,1)	
Glucose-2						
1‴	4.56	*d* (7.9)	105.59			
2‴	3.19	*dd* (9,8)	76.01			
3‴	3.37	*t* (9)	78.23			
4‴	3.31	*t* (9)	71.49			
5‴	3.25	*ddd* (9,6,2)	77.98			
6‴	3.68	*dd* (11,2)	62.70			
	3.84	*dd* (11,6)				
Rhamnose						
1⁗	4.64	*brs*	102.00	4.66	*brs*	102.35
2⁗	3.81	*dd* (3,1)	71.81	3.81	*dd* (3,1)	72.03
3⁗	3.83	*dd* (9,3)	72.42	3.63	*dd* (9,3)	72.62
4⁗	3.54	*t* (9)	77.98	3.33	*t* (9)	74.08
5⁗	3.59	*m*	68.44	3.57	*m*	69.95
6⁗	1.19	*d* (6.2)	18.05	1.16	*d* (6.2)	18.04

Cyanidin 3-[2″-(glucosyl)-6″-(rhamnosyl) glucoside] has been found in raspberry, raspberry–blackberry hybrids, tart cherry, red currant (Harborne and Hall, 1964) and boysenberry (McGhie et al., 2006) fruits, the leaves of *Begonia* and the flower petals of *Clivia miniata* (Harborne and Hall, 1964). As far as we know, anthocyanins including a **1**–**3** Glc–Glc bond have been found only in *Delphinium* as cyanodelphin (Kondo et al., 1991) and in *Allium* as Cy 3-laminaribioside (Terahara et al., 1994; Fossen et al., 1996, 2003; Donner et al., 1997). Cy 3-rutinoside, glucosylated at C-3 of Glc-1, has never been reported; thus, compound **1** (A1) is considered to be a novel anthocyanin.

2.2. Compound **2** (A2)

On UV–vis spectra analysis, three absorption maxima at 530, 331 and 282 nm for A2 were detected when acquired in 0.1% HCl–MeOH. Only Cy 3-glucoside was detected as a moderate hydrolysate from A2, indicating that the number of attached sugars was two. After acid hydrolysis, sugar residues were analyzed by TLC with aniline hydrogen phthalate reagent. Two spots were distinguished; one spot was identified as glucose and the other rhamnose. Co-chromatography of A2 with Cy 3-rutinoside obtained from snapdragon (*Antirrhinum majus*) established that compound **2** (A2) was the known Cy 3-rutinoside. This structure was further confirmed on the basis of its mass and NMR spectroscopic data.

2.3. Antioxidant activity

The relative ORAC values of compound **1** (A1) (3.92:1 mol Trolox equivalent/mol) and compound **2** (A2) (3.90) were larger than that of Cy 3-glucoside (2.71). It has been reported that cyanidin has the largest ORAC value (2.239 ± 0.029 μM Trolox equivalent/μM) among the anthocyanidins, because of its 3′,4′-di-OH substitution in the B ring (Wang et al., 1997). In addition, Cy 3-glucoside is

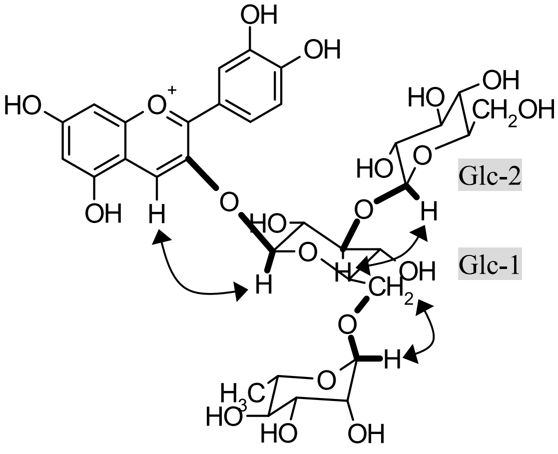

Fig. 1. Structure of anthocyanin A1 in purple asparagus with HMBC and NOE. The significant HMBCs are shown with bold lines. NOEs are shown with arrows.

one of the non-acylated cyanidin glycosides that have the largest ORAC values (Wang et al., 1997; Stintzing et al., 2002; Zheng and Wang, 2003; Aaby et al., 2004, 2007). It is therefore suggested that both compounds **1** (A1) and **2** (A2) have high antioxidant activity. Maeda et al. (2005) reported that purple asparagus was rich in rutin and polyphenols and that its DPPH (1,1-diphenyl-2-picrylhydrazyl) absorbing activity was high. As ORAC assays indicated that compounds **1** (A1) and **2** (A2) have high antioxidant activities, it is suggested that anthocyanin contributes to the high functionality of purple asparagus.

3. Conclusions

Two anthocyanins from the spears of purple asparagus (*A. officinalis*) were isolated and identified as cyanidin 3-[3″-(*O*-β-D-glucopyranosyl)-6″-(*O*-α-L-rhamnopyranosyl)-*O*-β-D-glucopyranoside] (**1**) and cyanidin 3-rutinoside (**2**), respectively. The former is considered to be a novel anthocyanin. ORAC assays suggest that they have high antioxidant activities.

4. Experimental

4.1. General

TLC was carried out on cellulose plates (pre-coated TLC plates CEL 400–10, 10 × 20 cm, Chemco) using *n*-BuOH–HOAc–H_2O (4:1:2 or 7:1:2 upper phase) and HOAc–HCl–H_2O (3:1:8) solvents. Analytical HPLC was performed on a C-R6A Chromatopac, SCL-6A system controller, SPD-6AV UV–vis spectrophotometric detector, LC-6A liquid chromatograph and a CTO-10A column oven (Shimadzu Corporation, Japan), using a Cosmosil $5C_{18}$ MS II packed column (250 mm × 4.6 mm i.d., Nacalai Tesque, Japan) at 40 °C with a flow rate of 1.0 ml/min, monitored at 530 nm. The solvent system was a linear gradient elution from 20 to 59% solvent B [H_3PO_4–HOAc–CH_3CN–H_2O (1.5:20:25:53.5)] in solvent A [H_3PO_4–H_2O (1.5:98.5)] for 24 min. Preparative HPLC was performed using a C-R6A Chromatopac, SCL-10A*VP* system controller, SPD-10AV*VP* UV–vis detector, LC-6AD liquid chromatograph and CTO-10A*VP* column oven (Shimadzu Corporation, Japan), with a Cosmosil $5C_{18}$ AR II, 250 mm × 20 mm i.d. packed column (Nacalai Tesque, Japan) at 35 °C and a flow rate of 9.0 ml/min, monitored at 520 nm. The solvent system was a linear gradient elution from 25 to 50% solvent B [HCO_2H–CH_3CN–H_2O (1:4:5)] in solvent A [HCO_2H–H_2O (1:9)] for 23 min.

UV–vis spectra were measured from 700 to 200 nm using a V-530 UV/VIS Spectrophotometer (JASCO, Japan) in 0.1% HCl–MeOH. High-resolution electron spray ionization mass spectra were recorded on a Q-FT-ICR MS APEX-Q94e (BRUKER Daltonics, Germany) with an Apollo II Dual Source in the positive mode. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using the positive ion reflector mode, Voyager-DE (Applied Biosystems, USA). 2,4,6-Trihydroxyacetophenon monohydrate (THAP) saturated in acetone was used as the matrix.

^1H NMR, ^{13}C NMR, $^1H\{^{13}C\}$ HSQC, $^1H\{^{13}C\}$ HMBC, TOCSY, DOF-COSY, NOESY and ROESY spectra were obtained on a DMX-750 spectrometer (BRUKER BIOSPIN, Germany). A1 and A2 were dissolved in 10% (v/v) TFA-d/CD_3OD mixture. The residual proton peaks and ^{13}C peaks of CD_3OD were used as internal standards (δ 3.30 for ^1H; 48.97 for ^{13}C).

4.2. Plant material

A. officinalis cv. Purple Passion grown in the outdoor field in Nagano Prefecture was used for this experiment. The fresh spears were collected from May 2006 to June 2006, and provided for anthocyanin purification.

4.3. Isolation and identification

Fresh peels of purple asparagus spears (1 kg) were incubated overnight in 4 l of HCO_2H–H_2O (1:9) at room temperature. After filtration, the filtrate was passed through a Diaion HP-20 resin column (700 mm × 60 mm i.d.) with 4 l of H_2O. The column was thoroughly washed with HCO_2H–H_2O (5:95) (8 l) and the anthocyanins were eluted by HCO_2H–MeOH (5:95). After concentration of the eluates, they were again purified with a Sephadex LH-20 column (700 mm × 50 mm i.d.) by HCO_2H–EtOH–H_2O (1:10:9). The eluate was concentrated and filtered. The anthocyanins were separated into two bands by ascending PC (ADVANTEC TOYO No. 526, Toyo Roshi) with *n*-BuOH–HCO_2H–H_2O (4:1:2) as the solvent. Several separated anthocyanins were collected by descending PC with HCO_2H–H_2O (1:9). The solution was concentrated and filtered through a HLC-DISK (25 Hydrophobic, φ0.45 μm, Kanto Chemical Co., Inc.). Anthocyanins were separated by HPLC (details are shown in Section 4.1). After EtO_2 was added, the precipitate was collected by centrifugation at 3000 rpm and dried under reduced pressure. The obtained anthocyanin powder was used for the experiments described below.

Each anthocyanin was dissolved in HCl–MeOH (1:99) and hydrolyzed with NaOH–H_2O (1:9) under N_2 for 45 min at room temperature. The deacylated anthocyanins were analyzed by HPLC.

Each anthocyanin was dissolved in 0.2 ml HOAc–H_2O (1:1) and an equal volume of 2 N HCl was added. The solution was then heated at 70–80 °C for 0, 5, 10, 30, 60, 120 or 210 min. The reaction products were analyzed by TLC and HPLC.

Each anthocyanin was also dissolved in 6 N HCl. The solution was heated at 100 °C for 60 min and anthocyanidin was extracted by isoamylalcohol. The resulting alcohol layer was analyzed by HPLC and TLC for the identification of anthocyanidin. The aqueous layer was dried under vacuum and the residue was re-dissolved in H_2O for sugar analysis by TLC with *n*-BuOH–HOAc–H_2O (4:1:2) as the solvent. The sample spots were detected by spraying aniline hydrogen phthalate reagent (100 ml *n*-BuOH saturated by H_2O, 0.96 g aniline and 1.66 g phthalic acid) and heating. Glucose, galactose, xylose, arabinose and rhamnose were used as authentic standards.

4.3.1. *Cyanidin 3-[3″-(O-β-D-glucopyranosyl)-6″-(O-α-L-rhamnopyranosyl)-O-β-D-glucopyranoside] (**1**)*

Red powder; UV–vis (nm): 282, 332 and 530; HR-FT-ICR MS: *m*/*z* 757.21880 (calc. for $C_{33}H_{41}O_{20}$: 757.21857); for ^1H and ^{13}C NMR spectroscopic data: see Table 1.

4.3.2. *Cyanidin 3-rutinoside (**2**)*

Dark red powder; UV–vis (nm): 282, 331 and 530; HR-FT-ICR MS: *m*/*z* 595.16576 (calc. for $C_{27}H_{31}O_{15}$: 595.16575); for ^1H and ^{13}C NMR spectroscopic data: see Table 1.

4.4. ORAC assay using fluorescein as the fluorescent probe

The ORAC assay was carried out following the method of Ou et al. (2001) with a slight modification. Fluorescein and a peroxyl radical generator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), were prepared in a 75 mM phosphate buffer at pH 7.4. Fifty microliter of sample and 50 μl of 0.25 μM fluorescein were placed in a 96-well microplate and the reaction was started by adding AAPH solution (100 μl, 50 mM). The phosphate buffer and Trolox were used as a blank and a standard, respectively. Fluorescence readings were performed every 2 min, with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

The relative ORAC values were calculated using the differences in the areas under the decay curves and were expressed as Trolox equivalents.

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