JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Isolation of Bilberry Anthocyanidin 3-Glycosides Bearing *ortho*-Dihydroxyl Groups on the B Ring by Forming an Aluminum Complex and Their Antioxidant Activity

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ABSTRACT: Bilberry anthocyanin bearing an *ortho*-dihydroxyl group on the B ring was selectively isolated by complex formation with flavocommelin and aluminum ions (Al^{3+}). The interaction between the anthocyanins, flavocommelin, and Al^{3+} might have happened in a manner similar to rigid supramolecules, such as commelinin, protocyanin, and other complex pigments. Complex blue pigment (CP, 8.85 ± 0.26 mg) with Al^{3+} was formed from 18.6 mg (15 μmol) of bilberry pigment, 18.2 mg (30 μmol, 2.0 equiv) of flavocommelin, and 0.03 mL of 0.5 M aluminum chloride aqueous solution (15 μmol, 1.0 equiv), yielding 36.5 ± 0.7% recovery of anthocyanins in the complex after precipitation by ethanol. Consequently, anthocyanins bearing *ortho*-dihydroxyl groups on the B rings (delphinidin 3-glycosides, cyanidin 3-glycosides, and petunidin 3-glycosides) predominating (98.0 ± 0.2%) in the complex were selectively isolated in a dose-dependent manner by the addition of Al^{3+}, increasing the antioxidative activity of the mixture of anthocyanins.

KEYWORDS: *Anthocyanidin 3-glycoside, complex pigments, ortho-dihydroxyl selectivity, Al^{3+}, DPPH*

INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) fruit contains 15 different anthocyanidin 3-glycosides (An3G) and 5 anthocyanidins, as shown in Figure 1. Bilberry extracts have beneficial biological properties, such as antioxidant activity,[1−3] anticancer activity,[3,4] and improvement of night vision.[5] These physiological functions are reported to be due to anthocyanins. Furthermore, superoxide anion radical scavenging activity and the inhibitory effect of ultraviolet (UV) light irradiation on lipid peroxidation depends upon the number of hydroxyl groups on the B ring of anthocyanins.[6] In addition, plant extracts contain foreign substances, such as sugar, proteins, phenolic compounds, off-flavors from original plant materials, complicated byproducts, etc. These foreign substances may inhibit or interfere with the useful functions of food components. Therefore, as part of the food additive preparation in food technology, it would be desirable to remove undesired compounds or to add more valuable function at low cost.

One unique idea for isolation of anthocyanin is the formation of supramolecular pigment (SMP), as reported by Tamura et al.[7] and Kondo et al.[8] Thus, blue anthocyanin pigment from the blue flower petals of *Commelina communis* was reconstructed and isolated. The chemical structure was subjected to X-ray analysis and determined to be a stoichiometric self-assembled supramolecular pigment consisting of six malonylawobanin molecules, six flavocommelin (FC) molecules, and two magnesium atoms.[8,9] When all components are mixed in a neutral solution, chiral stacking of each component and metal complexation occur spontaneously to form a SMP,[8,9] linked by weak noncovalent bonds. In the formation of SMP, undesired components are strictly excluded from the SMP.[10] After this report, five SMPs were found in blue flowers: *C. communis*,[7−9,11,12] *Centaurea cyanus*,[13−15] *Salvia patens*,[16] *Salvia uliginosa*,[17] and *Nemophila menziesii*.[18]

It is considered that an essential requirement of the anthocyanin structure for SMP formation is an anthocyanidin 3,5-diglucoside with at least one acyl group, an optimally cinnamoyl derivative, at the position of 6-OH of the 3-*O*-sugar residue. However, acylated anthocyanins are not common in the plant kingdom.

On the other hand, cyanidin 3-glucoside and delphinidin 3-glucoside are more abundant in plant anthocyanins. Hydrangea sepals are blue, purple, and red, and delphinidin 3-glucoside forms complexes with co-pigments, 3-caffeoylquinic acid (neochlorogenic acid), 3-*p*-coumaroylquinic acid, and an Al^{3+} ion, resulting in blue.[19−23]

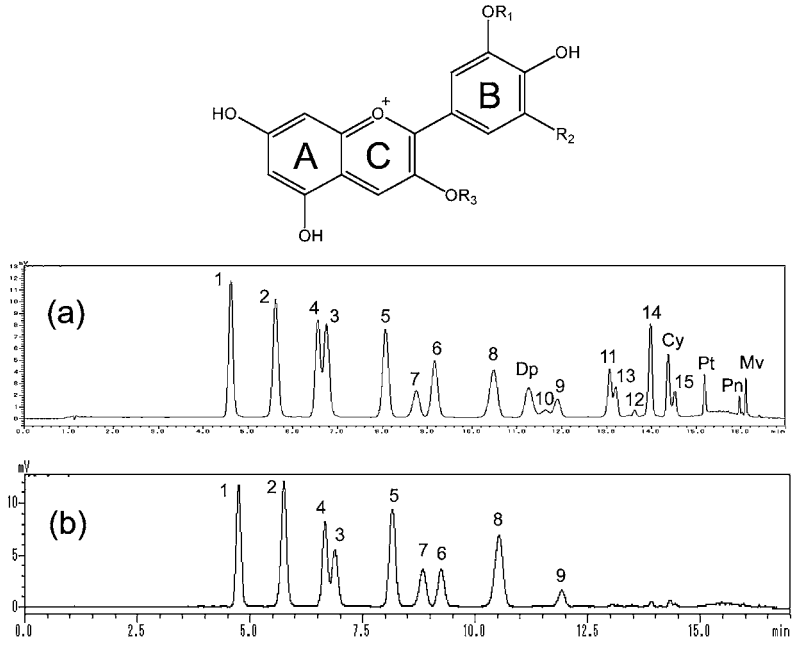
In this study, therefore, anthocyanidin 3-glycosides were considered for complex formation with phenolic compounds and metal ions. This trial may offer a method of producing pure anthocyanins without requiring any expensive equipment, resulting in an increase in the antioxidant activity of anthocyanins selected by SMP.

MARERIALS AND METHODS

Chemicals. Bilberry pigment (BILBERON-25) was obtained from Tokiwa Phytochemicals Co. (Chiba, Japan). Aluminum chloride, 25% ammonia solution, sodium carbonate, potassium chloride, phosphoric acid, acetic acid, 2-amino-2-hydroxymethyl-1,3-propanediol (tris(hydroxymethyl)aminomethane), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phenol reagent was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Gallic acid was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Trolox was purchased from EMD

Received: June 7, 2012
Revised: September 28, 2012
Accepted: October 1, 2012
Published: October 1, 2012

dx.doi.org/10.1021/jf302476n | *J. Agric. Food Chem.* 2012, 60, 10634−10640



Peak	Anthocyanin	R_1	R_2	R_3
1	Delphinidin 3-galactoside	H	OH	Galactose
2	Delphinidin 3-glucoside	H	OH	Glucose
3	Delphinidin 3-arabinoside	H	OH	Arabinose
4	Cyanidin 3-galactoside	H	H	Galactose
5	Cyanidin 3-glucoside	H	H	Glucose
6	Cyanidin 3-arabinoside	H	H	Arabinose
7	Petunidin 3-galactoside	H	OCH_3	Galactose
8	Petunidin 3-glucoside	H	OCH_3	Glucose
9	Petunidin 3-arabinoside	H	OCH_3	Arabinose
10	Peonidin 3-galactoside	CH_3	H	Galactose
11	Peonidin 3-glucoside	CH_3	H	Glucose
12	Peonidin 3-arabinoside	CH_3	H	Arabinose
13	Malvidin 3-galactoside	CH_3	OCH_3	Galactose
14	Malvidin 3-glucoside	CH_3	OCH_3	Glucose
15	Malvidin 3-arabinoside	CH_3	OCH_3	Arabinose

Figure 1. Structure and chromatogram of bilberry (a) anthocyanins and (b) CP. Dp, Delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; and Mv, malvidin (aglycones). The analytical condition of UFLC is described in the Materials and Methods with Shimadzu LC-20AV equipped with a Shim-pack XR-ODS column (100 × 3.0 mm inner diameter; 2.2 μm) at 520 nm.

Biosciences, Inc. (Darmstadt, Germany). Methanol (MeOH), ethanol (EtOH), and acetonitrile were used after distillation. Barnstead E-pure (>17.8 Ω) (Hansen and Co.) was used for distilled water. FC was isolated from *C. communis* and purified by crystallization with acetonitrile and water.

Formation of Complex Pigments. Bilberry pigment was dissolved in MeOH (18.6 mg/200 μL) and neutralized with 0.5 mL of 1 N NH_3 aqueous solution. The color of this solution changed from red to bluish purple. The reaction mixture was immediately evaporated to dryness in a vacuum to remove excess NH_3. The residue was mixed with an aqueous solution of FC (18.2 mg/300 μL) and 30 μL of 0.5 M aluminum chloride aqueous solution. The reaction mixture was allowed to stand for 10 min and evaporated to dryness in a vacuum. The residue was dissolved in 0.3 mL of water, and then EtOH (2.1 mL) was added 7 times to the solution. This mixture was allowed to stand at −20 °C overnight, whereby a part of the pigment formed the precipitates. This mixture was centrifuged to separate the precipitates. The precipitates [complex pigments (CP)] were dried in a desiccator with $CaCl_2$ for several days. Its UV−visible (vis) spectrum was recorded on a JASCO V-520-SR UV−vis spectrometer (JASCO Corp., Tokyo, Japan). Circular dichroism (CD) was measured with a JASCO J-20C spectrometer (JASCO Corp., Tokyo, Japan).

Purification of Complex Pigments by Sephadex G-10. A total of 50 mg of CP was dissolved in 0.5 mL of water. This solution was applied to a Sephadex G-10 column (15.0 × 1.0 cm inner diameter) and eluted by water. Fractions (fractions 1−3) were separated by difference in color of solutions. A deep blue fraction among the eluents (fraction 1) was collected and evaporated to dryness in a vacuum. Crude pigment from fraction 1 was dissolved in a minimum amount of water (0.2 mL), and then EtOH (0.6 mL) was added 3 times. This mixture was allowed to stand at −20 °C overnight to make the precipitates of the pigment at the lower temperature and then centrifuged to obtain CP. The CP was

dried in a desiccator with $CaCl_2$ for several days. The yields are described in Table 3.

Stability Test. Each sample of bilberry, commelinin, and CP was dissolved in various buffer solutions at pH 3.0, 5.6, and 7.0, and then distilled water was used to adjust the concentration to 1.4 mg/mL. These solutions were stored in a dark place at 25 °C for different periods of time (0, 1, 3, and 12 h, and 1, 3, and 7 days). Color stability was determined using an UV−vis spectrophotometer (JASCO V-520-SR) at the wavelength of maximum absorbance (λ_{max}) of each sample by a 1 cm length of a quartz cell

$$\text{color stability (\%)} = \text{Abs}_t/\text{Abs}_0 \times 100$$

where Abs is the absorbance and t is the time after dissolving the pigment.

Color Value. The color value means the purity of colorant, standardized by absorbance of the colorant of 10% (w/v) (1 g/10 mL) solution at the wavelength of maximum absorption (λ_{max}). Generally, the color value was expressed as $E^{10\%}_{1\ cm}$ in citric buffer (pH 3.0), and absorbance was determined using an UV−vis spectrophotometer (JASCO V-520-SR) at 520 nm by a 1 cm length of a quartz cell.[24]

$$\text{color value}=\{0.1/\text{sample amount (g)} \times \text{media volume (mL)}\} \times \text{Abs}$$

Quantitative amounts of anthocyanins were calculated from the calibration curve of cyanidin 3-glucoside.

Total Phenol Content (TP). TP was measured according to the Folin−Ciocalteu method.[25] Each sample (bilberry, supernatant, precipitation, and FC) was dissolved in distilled water at a concentration of 1 mg/mL, and then 0.1 mL of these solutions, 1 mL of 50% phenol reagent, 1 mL of 10% sodium carbonate aqueous solution, and 4 mL of distilled water were mixed in this order. After this reaction solution was stored in a dark place for 1 h at room temperature, absorbance was determined at 760 nm by a 5 mm length of a quartz cell using an UV−vis spectrophotometer (JASCO V-520-SR). To make a standard curve, gallic acid standard solutions (0, 200, 400, 600, 800, and 1000 μg/mL) were measured in the same manner described above. The amount of TP in each sample was calculated to gallic acid equivalent (μg/mL of GAE).

DPPH Radical Scavenging Activity (DRSA). DRSA was the method used to determined a decrease in the absorbance of DPPH free radical by measuring the visible absorbance.[26] EtOH, 0.9 mM DPPH EtOH solution, and 0.05 M Tris-HCl buffer were mixed with 1:1:1, and then 3 mL of this mixture solution, 0.5 mL of Tris-HCl buffer, and 0.5 mL of each sample solution (same as TP, 1 mg/mL) were mixed in order. After this reaction solution was stored in a dark place for 30 min at room temperature, absorbance was determined at 525 nm by a 5 mm length of a quartz cell using an UV−vis spectrophotometer (JASCO V-520-SR). To make a standard curve, the antioxidation activity of a Trolox standard solution (0.4 mM Trolox solution was added. The content of Trolox (0, 80, 160, 240, 320, and 400 nmol) was measured in a similar way.

Chromatography. Analytical ultrafast liquid chromatography (UFLC) was carried out using a Shimadzu LC-20AD gradient system under the following conditions: Shim-pack XR-ODS column (100 × 3.0 mm inner diameter, 2.2 μm), temperature maintained at 40 °C, detector Shimadzu SPD-20AV set at λ = 280 and 520 nm, mobile phase A of AcOH/CH_3CN/H_2O/H_3PO_4 (4.0:5.0:89.5:1.5), and mobile phase B of AcOH/CH_3CN/H_2O/H_3PO_4 (14.0:17.5:67.0:1.5). The elution conditions are described as follows: isocratic elution of 0% B, 0−1.5 min; linear gradient from 0 to 15% B, 1.5−9.5 min, linear gradient from 15 to 50% B, 9.5−13.0 min, linear gradient from 50 to 100% B, 13.0−14.0 min, isocratic elution of 100% B, 14.0−16.0 min, and linear gradient from 100 to 0% B, 16.0−16.2 min, with a flow rate of 0.5 mL/min and injection volumes of 1 μL.

Quantitative Analysis of Aluminum by Inductively Coupled Plasma (ICP) Emission Spectroscopy. ICP emission spectroscopy was carried out using a SRS1700VR ICP emission spectrophotometer (SII Nano Technology, Inc. Chiba, Japan). Each sample (supernatant, CP, purified CP by Sephadex G-10, and separated An3G from CP) was dissolved in 0.1 N nitric acid aqueous solution at a concentration of 250 ppm.

RESULTS AND DISCUSSION

Formation of CP. When bilberry anthocyanins [anthocyanidin 3-glycoside (An3G)], FC, and aluminum ions (Al^{3+}) were mixed in an aqueous solution, similar to the method for commelinin,[7,10] blue complex precipitates (CP) composed of An3G, FC, and Al^{3+} were obtained by ethanol precipitation. As shown in Table 1, the yields of CPs formed by different amounts of Al^{3+} (5, 15, 25, and 50 μmol) were 4.39, 8.85, 1.12, and 0.12 mg, respectively. The ratios of An3G/FC in the precipitates with different amounts of Al^{3+} (5, 15, 25, and 50 μmol) were 1:0.9, 1:0.9, 1:0.8, and 1:2.3, respectively. With the exception of the 50 μmol addition of Al^{3+}, the An3G/FC ratios were almost consistent at around 1:0.8 on a per mole basis, even though FC was added to An3G 2 times on a per mole basis. These CPs kept a constant ratio of An3G/FC in the precipitates, even though the ratios of An3G/Fc and Al^{3+} contents were changed in the different concentrations at the beginning concentration of CP formation. The complex force may not be weak. Additionally, the recovery rates of An3G in the CPs formed by different amounts of Al^{3+} (5, 15, 25, and 50 μmol) were 19.0, 36.5, 39.0, and 0.6%, respectively, after the amounts of An3G were estimated from the UV−vis absorption (λ_{max}) of cyanidin 3-glucoside isolated from Delaware Grape [ε = 15 200 (283 nm) and 26 000 (529 nm)] and then listed in Table 1 as milligram units. At high concentrations of Al^{3+}, recovery of An3G as precipitates was quite low (0.6%) because of the higher solubility of An3G in the supernatant.

Table 1. Amount of Supernatant and Precipitate after Complexes with Different Amounts of Aluminium Ions[a]

	amount (mg)			
	addition of aluminum ions (μmol)			
	5	15	25	50
supernatant	28.39	31.36	39.86	45.14
anthocyanins[b]	2.27	2.24	2.79	4.51
FC	15.05	10.29	10.40	15.30
An3G/FC[c]	1:4.9	1.0:3.1	1.0:2.8	1.0:2.5
precipitate	4.39	8.85	1.12	0.22
anthocyanins[b]	1.36	2.91	0.37	0.04
FC	1.62	3.69	0.39	0.13
An3G/FC[c]	1.0:0.9	1.0:0.9	1.0:0.8	1.0:2.3

[a]The complex was formed by mixing 18.6 mg of bilberry pigment (containing 6.81 mg of anthocyanins, 15 μmol) with 18.2 mg of FC (30 μmol, 2 equiv) and 0.5 M aluminum chloride aqueous solution. [b]The anthocyanin content was estimated as the cyanidin 3-glucoside (ε = 26 000) standard curve. [c]An3G/FC is expressed as a mole ratio.

Table 2 shows the types of anthocyanidin and the composition of An3G in bilberry fruit and CPs. The compositions of An3G bearing an *ortho*-dihydroxyl group on the B ring in CP were 96.1, 97.6, 98.6, and 85.1%, respectively, being highly selective and dose-dependent on Al^{3+} without the CP prepared by the addition of 50 μmol of Al^{3+}. The more Al^{3+} increased, the more the yield of CP and selectivity of An3G bearing an *ortho*-dihydroxyl group on the B ring decreased. At 5 μmol of Al^{3+}, the amounts of delphinidin 3-glycosides (Dp3G) and petunidin 3-glycosides (Pt3G) were higher than those of the original bilberry pigment. However, the amount of cyanidin 3-glycosides (Cy3G) was smaller than that of the original bilberry pigment. We surmise that Al^{3+} might quickly form the complex precipitates of Dp3G and Pt3G rather than Cy3G because of the number of

Table 2. Composition of Anthocyanins in Supernatant and Precipitation after Complexes with Different Amounts of Aluminium Ions[a]

	composition of anthocyanins[b] (%)								
		addition of aluminum ions (μmol)							
		5		15		25		50	
	bilberry	Sup[c]	Pre[d]	Sup[c]	Pre[d]	Sup[c]	Pre[d]	Sup[c]	Pre[d]
Dp3G	37.7	24.8	51.5	27.6	46.1	37.4	40.8	38.1	38.9
Cy3G	31.0	29.8	27.5	26.0	32.1	29.3	33.3	29.9	29.9
Pt3G	14.5	12.4	17.1	8.9	19.4	13.5	24.5	14.5	16.5
Sub1[e]	83.2	67.0	96.1	62.5	97.6	80.2	98.6	82.5	85.1
Pn3G	4.8	11.2	1.2	12.8	0.9	7.0	0.5	5.9	5.0
Mv3G	12.0	21.8	2.7	24.7	1.5	12.8	0.9	11.6	9.7
Sub2[f]	16.8	33.0	3.9	37.5	2.4	19.8	1.4	17.5	14.7

[a]The complex was formed by mixing 18.6 mg of bilberry pigment (anthocyanins, 15 μmol) with 18.2 mg of FC (30 μmol, 2 equiv) and 0.5 M aluminum chloride aqueous solution. [b]The composition of anthocyanins unified the same aglycones. [c]Sup = supernatant. [d]Pre = precipitation. [e]Sub1 = anthocyanins bearing *ortho*-dihydroxyl groups on the B ring. [f]Sub2 = anthocyanins expect Sub1 containing bilberry pigment.

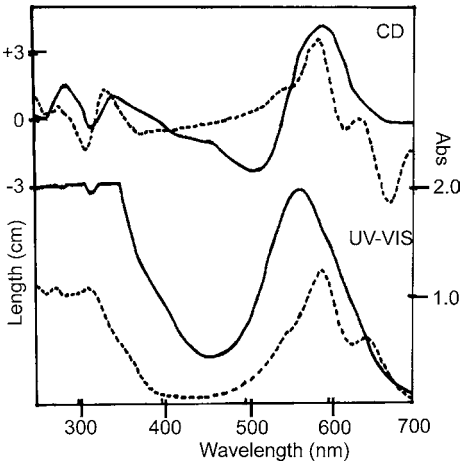


Figure 2. UV−vis and CD spectra of each complex. Commelinin was dissolved in water at a concentration of 0.9 mg/mL. CP was dissolved in the water concentration at 1.8 mg/mL. Commelinin, dashed line; CP, solid line.

substituents and electron densities on the B ring of those An3G. Consequently, the appropriate amount of Al^{3+} against the anthocyanin concentration for CPs was restricted and essential to form a highly selective formation of CPs. At the optimal CP conditions of anthocyanin (15 μmol), 2.0 equiv of FC and 1.0 equiv of Al^{3+} showed the highest recovery of CP, 8.85 ± 0.26 mg (recovery rate of anthocayanins, 36.5 ± 0.7%; selectivity, 98.0 ± 0.2%) in triplicates. A typical chromatogram of CP from 18.6 mg of bilberry pigment (15 μmol of An3G), 30 μmol of FC, and 15 μmol of Al^{3+} is shown in Figure 1. Five kinds of anthocyanidins disappeared from the precipitates. Moreover, compounds **1**−**9**, each bearing an *ortho*-dihydroxyl group on the B ring, were detected, but other compounds decreased or disappeared altogether. Selective formation of CPs is observed in this chromatogram.

On the CD spectrum of commelinin, one kind of natural SMP shows a strong negative exciton-type Cotton curve, but the CD spectrum of CP showed a positive exciton-type Cotton curve in Figure 2. This shape of CD was very close to that of the cyanidin 3,5-diglucoside and Al^{3+} complex reported by Goto et al.[27] However, this CP actually contained FC (An3G/FC = 1:0.8) as the essential component.

Purification of CP. CP prepared in the ratio of An3G/FC/Al^{3+} = 1:2:1 formed the rigid ratio of An3G/FC/Al^{3+} = 1:0.8:0.9 after purification by Sephadex G-10 column chromatography. Deep blue pigments were eluted as the first eluent and the main fraction of pigment without any interaction with Sephadex G-10 (lowest exclusion is 700). Therefore, An3G and FC could be retained and separated by the molecular sieve effect of the

Table 3. Composition of Anthocyanins in CP by Different Purifying Methods

	composition of anthocyanins (%)					
				EtOH reprecipitation (water/EtOH)		
	bilberry	CP[a]	Sephadex G-10[b]	1:1	1:3	1:5
Dp3G	37.7	42.6	45.8 ± 0.5	45.3 ± 0.2	45.5 ± 0.1	45.6 ± 0.1
Cy3G	31.0	31.9	33.8 ± 0.3	33.9 ± 0.1	33.7 ± 0.1	33.8 ± 0.03
Pt3G	14.5	16.7	19.5 ± 0.2	19.2 ± 0.3	19.2 ± 0.1	19.2 ± 0.1
Pn3G	4.8	2.5	0.4 ± 0.01	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.02
Mv3G	12.0	6.3	0.5 ± 0.06	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
purity[c] (%)	36.6	20.8	44.2 ± 3.1	39.3 ± 1.5	37.9 ± 7.0	34.2 ± 2.1
recovery rate[d] (%)			67.3 ± 2.9	54.0 ± 3.6	81.6 ± 17.6	85.5 ± 6.4
An3G/FC[e]		1:1.7	1:0.7 ± 0.1	1:0.9 ± 0.1	1:0.8 ± 0.1	1:0.8 ± 0.1

[a]To form CP, 1.5 mmol of aluminum was added to a mixture of 1.5 mmol of anthocyanins and 3.0 mmol of FC. [b]Deep blue elution (fraction 1) by Sephadex G-10 was evaporated and precipitated by the addition of ethanol (water/EtOH = 1:3). [c]The purity was estimated by the following equation: purity (%) = (amount of anthocyanin in CP/amount of CP) × 100. Amount of anthocyanins in CP was estimated as the cyanidin 3-glucoside (ε = 26000) standard curve. The purity maximum of anthocyanin in CP may be around 50%, because of CP contained FC. However, the purity maximum of bilberry pigment was 100%. [d]The recovery rate was estimated by the following equation: recovery rate (%) = {amount of anthocyanins in CP/10.9 (amount of anothocyanin in used CP, mg)} × 100. [e]An3G/FC is expressed as a mole ratio (n = 3).

column. Finally, blue pigments of CP were collected by adding three volumes of EtOH against the solution described in the Materials and Methods, and then the precipitate was collected by centrifugation and drying the eluents.

The purity of An3G prepared by Sephadex G-10 was compared to that prepared by different conditions of EtOH precipitation, as shown in Table 3. Sephadex G-10 showed the most selective separation of An3G bearing an *ortho*-dihydroxyl group on the B ring. CP without any purification contained 2.5% peonidin 3-glycosides (Pn3G) and 6.3% malvidin 3-glycosides (Mv3G), but after purification with the Sephadex G-10 column, only 0.4% Pn3G and 0.5% Mv3G were observed in the product. Higher purity (44.2% against around 50%) was also observed for Sephadex G-10 column chromatography. However, EtOH precipitation techniques also gave similar selective composition of An3G in CPs with different amounts of EtOH. The recovery rates with 3−5 times the volume of EtOH were quite high, but the purities of the EtOH precipitation technique were not high. We can conclude that Sephadex G-10 column chromatography and EtOH precipitation techniques practically offer high efficiency of isolation of An3G bearing an *ortho*-dihydroxyl group on the B ring.

With the practical isolation of An3G bearing an *ortho*-dihydroxyl group on the B ring, 1.86 g of bilberry pigment (An3G, 1.5 mmol, estimated from the calibration curve of cyanidin 3-glucoside) neutralized by ammonium aqueous solution was mixed with 1.82 g (2 equiv) of FC and 3 mL of 0.5 M aluminum chloride aqueous solution (Al^{3+}, 1 equiv) and stirred for 15 min. Finally, the precipitates formed in the solution by adding EtOH to obtain the CP, yielding 1.30 g, and the recovery rate of An3G in CP was 40.2%. The purity of An3G in the CP after purification with Sephadex G-10 and Discovery DPA-6S was 96.9%, and the recovery rate of An3G was 90.6%. Additionally, the composition of An3G bearing an *ortho*-dihydroxyl group on the B ring was 99.1%. After purification, the aluminum content was determined by an ICP emission spectrophotometer. Separated An3G from CP contained 0.05 ppm of aluminum in 250 ppm of separated An3G solution, and aluminum was reduced to 1:300 (mol ratio) against An3G. With this technique, the safety of food colorants with aluminum contamination can be guaranteed.

Table 4 shows the color value of bilberry, CP, purified CP by Sephadex G-10, and An3G separated from CP. The purity of CP was proven to be quite high because separated An3G from CP showed a color value of 1740 compared to 1880 for authentic cyanidin 3-glucoside.

As a result, An3G mixtures of Dp3G, Cy3G, and Pt3G bearing an *ortho*-dihydroxyl group on the B ring were selectively

Table 4. Color Value[a]

	color value
bilberry pigment	626 ± 21
CP	686 ± 51
purified CP by Sephadex G-10	1047 ± 33
anthocyanins separated from CP[b] (ε^c = 25 200 at 535 nm)	1740 ± 49
cyanidin 3-glucoside (ε^c = 26 000 at 529 nm)	1880

[a]The color value was estimated by the following equation: color value = {0.1/sample amount (g) × media volume (mL)} × Abs. [b]Anthocyanins were separated by Discovery DPA-6S column chromatography from CP. [c]ε was estimated by measured sample solution (0.01% HCl−MeOH, 12.5 mM) absorbance at λ_{max} by 1 cm length of a quartz cell.

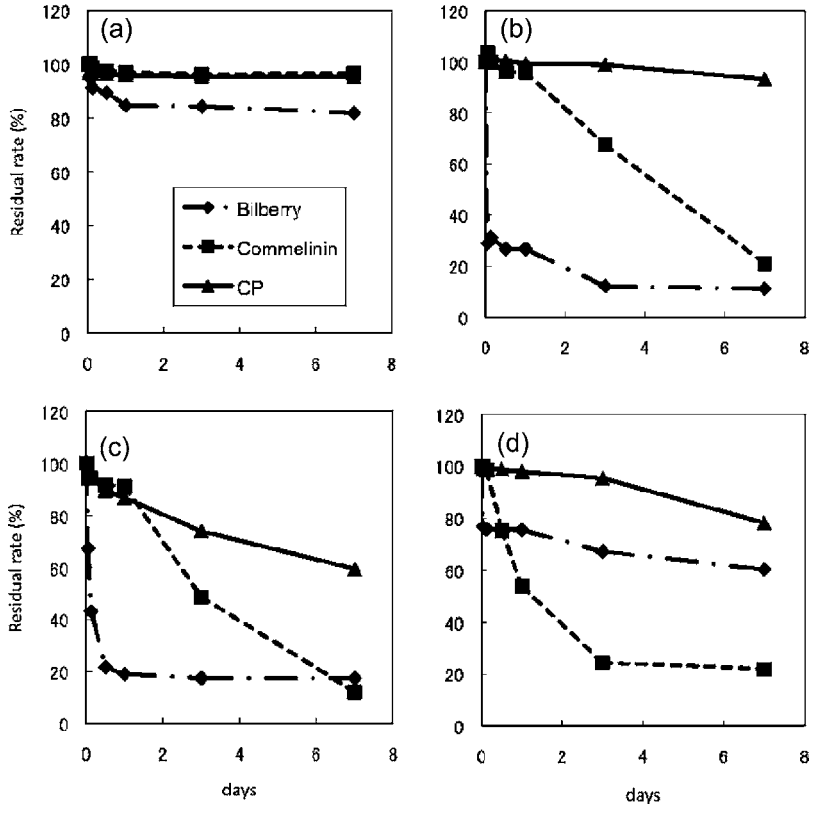


Figure 3. Stability test of CP_{Al} at difference pH values: (a) pH 3.0 citric buffer, (b) pH 5.6 acetate buffer, (c) pH 7.0 phosphate buffer, and (d) water. The sample concentration was 1.4 mg/mL and stored in a dark place at 25 °C. The residual rate was determined at λ_{max} of each sample. Bilberry, dashed-dotted line; commelinin, dashed line; CP, solid line.

dx.doi.org/10.1021/jf302476n | J. Agric. Food Chem. 2012, 60, 10634−10640

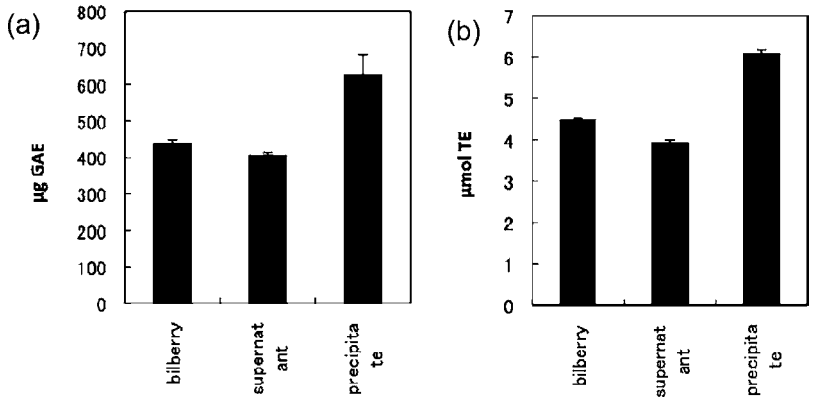


Figure 4. TP and DRSA of anthocyanins separated by column chromatography and precipitation from bilberry pigment, supernatant, and precipitate (CP). The sample concentration was 1.0 mg/mL. (a) TP and (b) DRSA (mean ± SD; n = 3).

precipitated in ethanolic aqueous solution and eluted at the higher molecular weight fraction of Sephadex G-10. The isolated CP also showed a positive exciton-type Cotton curve. An3G, An3G + FC, and An3G + Al^{3+} did not show a similar exciton type of Cotton curve to CP. This evidence partly proves the Al^{3+} complex of bilberry anthocyanins and FC. More detailed data of the solid chemical structure of CP will be proven in the near future with simpler anthocyanin-containing plant pigments.

Additionally, because FC can be recrystallized easily after usage in this experiment, this new approach for the purification will provide a technique for the isolation of a large amount of specific anthocyanins with a low cost of performance.

Stability of CP. It is well-known that anthocyanins can form different chemical structures, such as flavylium cation (red), carbinol pseudo-base (colorless), quinoidalbase (blue−purple), and chalcone (yellow), depending upon the pH.[28] Generally, anthocyanins have been used as food colorants at lower pH, because the flavylium cation form becomes dominant, and then the form is the most stable. The stability of CP formed by bilberry pigment, FC, and Al^{3+} was compared to bilberry pigment and commelinin, a natural complex pigment. As the results show, CP has higher color stability than the original bilberry pigment and commelinin at pH values of 3.0, 5.6, 7.0, and water (Figure 3). At pH 3.0, An3G retained more than 95% of its color 1 week after dissolving. At low pH, CP might maintain a higher stability by co-pigmentation of the flavylium form of An3G with FC. On the other hand, at higher pH (5.6, 7.0, and water), CP still kept its original color after being dissolved in those solutions. Therefore, the stability of CP significantly increased and improved the practical usage of the pigments.

TP and DRSA. Figure 4 shows TP and DRSA of An3G isolated by column chromatography from each sample (bilberry pigment, supernatant, and CP). TPs were 440.6 ± 8.6 μg/mg of GAE for An3G from original bilberry pigment, 407.1 ± 5.9 μg/mg of GAE for An3G from the supernatant, 628.4 ± 53.3 μg/mg of GAE for An3G from the CP, and 40.0 ± 1.2 μg/mg of GAE for pure FC. Furthermore, DRSAs were 4491.1 ± 24.9 nmol/mg of Trolox equivalent (TE) for An3G from original bilberry pigment, 3936.1 ± 53.1 nmol/mg of TE for An3G from the supernatant, 6080.0 ± 88.0 nmol/mg of TE for An3G from the CP, and 33.2 ± 2.4 nmol/mg of TE for pure FC. An3Gs bearing an *ortho*-dihydroxyl group on the B ring of bilberry pigment, supernatant, and CP were 83.6, 47.5, and 94.8%, respectively. An3G bearing an *ortho*-dihydroxyl group on the B ring of CP was 1.1 times higher than that of the original bilberry pigment. However, TP and DRSA of CP increased 1.4 times more than original bilberry pigment. These results show TP and DRSA increased according to the number of *ortho*-dihydroxyl groups on the B ring. Thus, our separation technique for An3G bearing an *ortho*-dihydroxyl group on the B ring by CP formation might offer one advanced technology to obtain some functional anthocyanins.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Nobuyoshi Asahi from the Research Center for Industrial Science and Technology (RIST) Kagawa for performing ICP emission spectroscopy analysis.

ABBREVIATIONS USED

UFLC, ultrafast liquid chromatography; DPPH, 1,1-diphenyl-2-picrylhydrazyl; An3G, anthocyanidin 3-glycosides; Dp3G, delphinidin 3-glycosides; Cy3G, cyanidin 3-glycosides; Pt3G, petunidin 3-glycosides; Pn3G, peondin 3-glycosides; Mv3G, malvidin 3-glycosides; DRSA, DPPH radical scavenging activity; TP, total phenol content; GAE, gallic acid equivalent; TE, Trolox equivalent; Abs, absorbance; UV, ultraviolet; vis, visible; CD, circular dichroism; FC, flavocommelin; ICP, inductively coupled plasma

REFERENCES

(1) Kähkönen, M. P.; Heinämäki, J.; Ollilainen, V.; Heinonen, M. Berry anthocyanins: Isolation, identification and antioxidant activities. *J. Sci. Food Agric.* **2003**, *83*, 1403−1411.

(2) Yue, X.; Xu, Z. Changes of anthocyanins, anthocyanidins and antioxidant activity in bilberry extract during dry heating. *J. Food Sci.* **2008**, *73*, C494−499.

(3) Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, *51*, 68−75.

(4) Cooke, D.; Schwarz, M.; Boocock, D.; Winterhalter, P.; Steward, W. P.; Gescher, A.; Marczylo, T Effect of cyanidin 3-glucoside and anthocyanidin mixture from bilberry on adenoma development in the Apc^{Min} mouse model of intestinal carcinogenesis—Relationship with tissue anthocyanin levels. *Int. J. Cancer* **2006**, *119*, 2213−2220.

(5) Canter, P. H.; Ernst, E. Anthocyanosides of *Vaccinium myrtillus* (bilberry) for night vision—A systematic review of placebo-controlled trials. *Surv. Ophthalmol.* **2004**, *49*, 38−50.

(6) Tsuda, T.; Shiga, K.; Ohshima, K.; Kawakishi, S.; Osawa, T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochem. Pharmacol.* **1996**, *56*, 1033−1039.
(7) Tamura, H.; Kondo, T.; Goto, T. The composition of commelinin, a highly associated metalloanthocyanin present in the blue flower petals of *Commelina communis*. *Tetrahedron Lett.* **1986**, *27* (16), 1801−1804.
(8) Goto, T.; Kondo, T. Structure and molecular stacking of anthocyanins—Flower color variation. *Angew. Chem., Int. Ed.* **1991**, *30*, 17−33.
(9) Kondo, T.; Yoshida, K.; Nakagawa, A.; Kawai, T.; Tamura, H.; Goto, T. Structural basis of blue-colour development in flower petals from *Commelina communis*. *Nature* **1992**, *358*, 515−518.
(10) Kondo, T.; Oyama, K.-I.; Yoshida, K. Chiral molecular recognition on formation of a metalloanthocyanin: A supramolecular metal complex pigment from blue flower of *Salvia patens*. *Angew. Chem., Int. Ed.* **2001**, *40*, 894−897.
(11) Hayashi, K.; Abe, Y.; Mitsui, S. Blue anthocyanin from flowers of *Commelina*, the crystallization and some properties thereof. *Proc. Jpn. Acad.* **1958**, *34*, 373−378.
(12) Takeda, K.; Hayashi, K. Metalo-anthocyanins. I. Reconstruction of commelinin from its components, awobanin, flavocommelin and magnesium. *Proc. Jpn. Acad.* **1977**, *53* (B), 1−5.
(13) Kondo, T.; Ueda, M.; Tamura, H.; Yoshida, K.; Isobe, M.; Goto, T. Composition of protocyanin, a self-assembled supramolecular pigment from the blue cornflower, *Centaurea cyanus*. *Angew. Chem., Int. Ed.* **1992**, *33*, 978−979.
(14) Kondo, T.; Ueda, M.; Isobe, M.; Goto, T. A new molecular mechanism of blue color development with protocyanin, a supramolecular pigment from cornflower, *Centaurea cyanus*. *Tetrahedron Lett.* **1998**, *39*, 8307−8310.
(15) Shiono, M.; Matsugaki, N.; Takeda, K. Structure of the blue cornflower pigment. *Nature* **2005**, *436*, 791.
(16) Takeda, K.; Yanagisawa, M.; Kifune, T.; Kinoshita, T.; Timberlake, C. F. A blue pigment complex in flowers of *Salvia patens*. *Phytochemistry* **1994**, *35*, 1167−1169.
(17) Mori, M.; Kondo, T.; Yoshida, K. Cyanosalvianin, a supramolecular blue metalloanthocyanin, from petals of *Salvia uliginosa*. *Phitochemistry* **2008**, *69*, 3151−3158.
(18) Yoshida, K.; Kondo, T. Flower color and anthocyanins. In *New Aspect of Anthocyanins Science—Development of Health Benefits and Practical Use*; Tsuda, T., Suda, I., Tsushida, T., Eds.; Kenpakusha: Tokyo, Japan, 2009; pp 54−58.
(19) Toyama-Kato, Y.; Yoshida, K.; Fujimori, E.; Haraguchi, H.; Shimizu, Y.; Kondo, T. Analysis of metal elements of hydrangea sepals at various growing stages by ICP−AES. *Biochem. Eng. J.* **2003**, *14*, 237−241.
(20) Yoshida, K.; Ito, D.; Shinkai, Y.; Kondo, T. Change of color and components in sepals of chameleon hydrangea during maturation and senescence. *Phytochemistry* **2008**, *69*, 3159−3165.
(21) Allen, R. C. Influence of aluminum on the flower color of *Hydrangea macrophylla*. DC. *Contrib. Boyce Thompson Inst.* **1943**, *13*, 221−242.
(22) Jurd, L.; Asen, S. The formation of metal and "co-pigment" complexes of cyanidin 3-glucoside. *Phytochemistry* **1966**, *5*, 1263−1271.
(23) Schreiber, H. D.; Swink, A. M.; Godsey, T. D. The chemical mechanism for Al^{3+} complexing with delphinidin; A model for the bluing of hydrangea sepals. *J. Inorg. Biochem.* **2010**, *104*, 732−739.
(24) The measurement method of color value. In *Manual of Food Additive Official Book*, 8th ed.; Tanimura, A., Ed.; Hirokawa Shoten: Tokyo, Japan, 2007; pp B91−B95.
(25) Folin, O.; Ciocaluteu, U. On tyrosine and tryptophan determinations protein. *J. Biol. Chem.* **1927**, *73*, 627−650.
(26) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.
(27) Goto, T.; Tamura, H.; Kondo, T. Chral stacking of cyanin and pelargonin—Soluble and insoluble aggregates as determined by means of circular dichroism. *Tetrahedron Lett.* **1987**, *47*, 5907−5908.
(28) Castañeda-Ovando, A.; Pacheco-Hernández, M. d. L.; Páez-Hernández, M. E.; Rodríguez, J. A.; Galán-Vidal, C. A. Chemical studies of anthocyanins: A review. *Food Chem.* **2009**, *113*, 859−871.