Preparative-Scale Isolation of Four Anthocyanin Components of Black Currant (*Ribes nigrum* L.) Fruits

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Four anthocyanin components of black currant, delphinidin 3-*O*-*β*-rutinoside (D3R), cyanidin 3-*O*-*β*-rutinoside (C3R), delphinidin 3-*O*-*β*-glucoside (D3G), and cyanidin 3-*O*-*β*-glucoside (C3G), were successfully isolated as crystalline forms on a preparative scale. In this process, selective hydrolysis of the glucosides (D3G and C3G) and rhamnosides (D3R and C3R) was achieved by treatment with *β*-glucosidase and hesperidinase (α-rhamnosidase), respectively, to improve resolution of anthocyanin components. Especially, selective conversion of the rutinosides into glucosides made the amounts of D3G and C3G increase about 4- and 7-fold, respectively. D3R, C3R, D3G, and C3G were isolated from enzymatic hydrolysates of black currant anthocyanins through Amberlite XAD-7HP absorption followed by preparative HPLC separation, and their crystals were obtained as the flavylium chloride.

Keywords: *Anthocyanin; blackcurrant; delphinidin 3-O-β-rutinoside; cyanidin 3-O-β-rutinoside; hesperidinase*

INTRODUCTION

In the past decade, anthocyanin-rich foods and preparations have attracted attention due to their health-promoting benefits in terms of reducing the risk of coronary heart disease and preventing some chronic diseases. For example, the French paradox of red wine (*1*) and the ophthalmic activity of bilberry extract (*2*) are well-known. The amounts and compositions of anthocyanins are distinctly dependent on their origins, and these factors affect the physiological properties of anthocyanin-containing products, such as the antioxidative activity (*3*), and the bioavailability in terms of intestinal absorption (*4*). Although a variety of chromatographic (*5*) and spectroscopic (*6*) methods are available for qualitative analysis of anthocyanins, preparative-scale isolation of these compounds remains a challenge.

A mixture of anthocyanins extracted from bilberry (*Vaccinium myrtillus* L.) is reported to have biologically and pharmacologically useful properties including ophthalmic activity (*2*, *7*), and it has been found to consist of 15 components (*8*). In our recent studies, we found that oral intake of a mixture of black currant anthocyanins (BCA), prepared from black currant fruits, had beneficial effects on visual functions (*9*) and brought about a significant improvement of transient refractive alteration induced by work at visual display terminals (VDT) (*10*). Black currant fruits are known to contain four kinds of anthocyanins as the major components: delphinidin 3-*O*-*β*-rutinoside (D3R), delphinidin 3-*O*-*β*-glucoside (D3G), cyanidin 3-*O*-*β*-rutinoside (C3R), and cyanidin 3-*O*-*β*-glucoside (C3G) (*11*, *12*). We are interested in the evaluation of the physiological activity of the individual components, as this is expected to provide useful information concerning the structure−activity relationship. However, in previous attempts at preparative-scale isolation of these components only low yields were obtained (*12*, *13*).

In this paper, we describe preparative-scale isolation of four anthocyanin components of black currant fruits. A key step in our method is treatment with *β*-glucosidase and hesperidinase, which selectively hydrolyze the 3-*O*-*β*-glucosidic and 6^G-*O*-α-rhamnosidic linkages of the anthocyanins into the aglycons and 3-glucosides, respectively. Through such treatment, four intact components in the concentrate were reduced to two components, 3-rutinosides and 3-glucosides, and this resulted in a good yield of the four components of BCA in a preparative-scale procedure.

MATERIALS AND METHODS

General. Contents are expressed as percent (w/w) on a dry matter basis, unless otherwise described. Soluble solid contents were measured as °Brix using a digital refractometer Rx-5000 (Atago Co., Ltd., Tokyo, Japan). Fast atom bombardment mass spectra (FAB-MS) were recorded on a JEOL JMS-DX-705L with glycerol as the mounting matrix. UV spectra were obtained in 0.1 N HCl using a Shimadzu UV-160 spectrometer. FAB-MS were recorded on a JEOL JMS-DX-705L with glycerol as the mounting matrix. ^{1}H NMR and ^{13}C NMR spectra were recorded in 0.1% (v/v) trifluoroacetic acid (TFA)/D_2O on a JEOL GX 400 spectrometer. The chemical shifts were referenced indirectly to Me_4Si by setting ^{1}H from HOD at 4.78 ppm and ^{13}C from 1,4-dioxane at 67.4 ppm. Thermogravimetry (TG) derivative thermogravimetry (DTG) measurement was performed at Toray Research Center (Tokyo, Japan), and the TG-DTG curves were recorded on a Shimadzu TG-40M thermoanalyzer from 30 to 300 °C under a stream of nitrogen (50 mL/min). The sample weight and heating rate were 10 ± 1 mg and 10 °C/min, respectively.

Materials. Commercially available black currant juice concentrate (65 °Brix), produced from fruits harvested in 1998, was purchased from Iprona A.G. (Lana, Italy). Four anthocyanin components were confirmed to be present in the juice as shown by the HPLC profile in Figure 1A, and the content of each component is summarized in Table 1. Hesperidinase (soluble hesperidinase, Tanabe No. 2) and *β*-glucosidase (crude, from almond) were purchased from Tanabe Seiyaku Co., Ltd.

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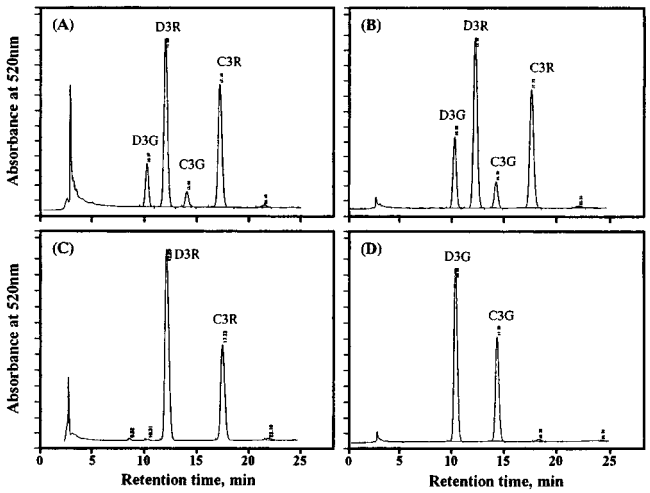

Figure 1. HPLC profiles of four kinds of BCA preparations during the isolation process: (A) commercial BC juice concentrate; (B) BCA concentrate; (C) mixture after β-glucosidase treatment; (D) mixture after hesperidinase treatment.

(Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively.

Concentration of BCA. Commercially available black currant juice concentrate (10 L, 46.2 g of total anthocyanins) was diluted to 20 °Brix by the addition of 0.2 N hydrochloric acid (22.5 L), and the resultant solution was applied to a column (∅ 11 cm × 42 cm) of cation-exchange resin (Amberlite 200CT, ∅ 0.50−0.65 mm, Rohm and Haas, Philadelphia, PA) followed by successive elution with 52 L each of 0, 25, and 50% (v/v) ethanol in 0.2 N hydrochloric acid. The fraction (52.2 L, 0.291 °Brix) obtained upon elution with 25% (v/v) ethanol was concentrated in vacuo to obtain a solid matter, the BCA concentrate (152 g), with the intact anthocyanin components accounting for 16.4 g (35.5% recovery in this step).

Treatment of BCA Concentrate with β-Glucosidase or Hesperidinase and Isolation of Four Anthocyanin Components. To a solution of BCA concentrate (40 g, 4.32 g of total anthocyanins) in 50 mM acetate buffer (1.5 L, pH 3.5) was added β-glucosidase [1000 units, 3.32 g in the same buffer (0.5 L)], and the mixture was stirred for 8 h at 40 °C. After the addition of 3% phosphoric acid (2.0 L), the resultant mixture was applied to a column (∅ 13 cm × 30 cm) of nonionic polymeric absorbant (Amberlite XAD-7HP, ∅ 0.35−0.90 mm, Rohm and Haas) followed by washing with 2.0 L of water containing 0.1% (v/v) TFA. Then, elution with aqueous MeOH (MeOH/H_2O = 80:20) containing 0.1% (v/v) TFA was performed, 0.2 L fractions of the eluate were collected, and the anthocyanin content was monitored by measuring the UV absorbance (at 520 nm). The anthocyanin-rich fractions (21−30) were pooled and evaporated in vacuo at 35 °C, resulting in a dark red syrup (26 mL, 46 °Brix, 3.43 g of anthocyanins). Twenty milliliter portions of the syrup (120 mL in total), adjusted to ~10 °Brix, were injected into a preparative HPLC system, operated at room temperature under the following conditions: column, ODS 120T (∅ 5.5 cm × 30 cm, Tosoh Co., Ltd., Tokyo, Japan); eluent, H_2O containing 9% (v/v) acetonitrile and 0.1% (v/v) TFA; flow rate, 80 mL/min; detector, UV (520 nm). Two large peaks appeared at 75−122 and 168−240 min, and the constituents eluted at 76−100 and 170−206 min were collected, resulting in two anthocyanin-rich fractions, 24.0 and 35.5 L, which were evaporated in vacuo to afford a dark red solid matter, 1.15 and 1.01 g, respectively. By recrystallization from methanol/5.0% hydrochloric acid (25:2), the former yielded fine deep purple crystals (0.97 g, D3R·Cl·$1.5H_2O$), and the latter yielded fine dark red crystals (0.76 g, C3R·Cl·$0.5H_2O$). Their physical and spectral properties are shown in Table 2.

BCA concentrate (40 g) in 50 mM acetate buffer (0.75 L, pH 3.5) was treated with hesperidinase [42.5 units, 79.5 g in the same buffer (0.25 L)] at 40 °C for 14 h, and the resultant mixture was subjected to treatment with 3% phosphoric acid followed by Amberlite XAD-7HP in the same manner as in the case of β-glucosidase treatment, resulting in a dark red residue (200 mL, 10 °Brix, 3.72 g of anthocyanins). Twenty milliliter portions of the syrup were injected into the preparative HPLC system under the same conditions as described above. Two large peaks appeared at 67−126 and 134−184 min, and the constituents eluted at 69−90 and 142−172 min were collected, resulting in two anthocyanin-rich fractions, 35.2 and 46.4 L, which were evaporated in vacuo to afford a dark red solid mass, 1.09 and 0.84 g, respectively. Recrystallization of the former and the latter from methanol/5.0% hydrochloric acid (25:2) yielded deep purple crystals (0.90 g, D3G·Cl·$0.5H_2O$) and dark red crystals (0.62 g, C3G·Cl·$0.5H_2O$), respectively, and their physical and spectral properties are shown in Table 2.

Analytical Methods. Analysis of anthocyanins was performed using an HP 1100 series HPLC system (Hewlett-Packard Co., Wilmington, DE) equipped with a Zorbax SB C-18 column (4.6 mm × 250 mm, particle size = 5 μm) and a photodiode array detector. Injection was performed by means of an autosampler with a 100 μL fixed loop, and the volume injected was 20 μL. Elution was performed using a solvent system consisting of a mixture of solvent A (0.5% phosphoric acid) and solvent B (methanol), applied as a linear gradient from 80% A/20% B (v/v) to 77% A/23% B (v/v) for 15 min and then held at 77% A/23% B (v/v) for a further 8 min, at a flow rate of 1.0 mL/min. The chromatograms were obtained with detection at both 280 and 520 nm. Each component peak was identified postrun through spectroscopic analysis by photodiode array detection from 200 to 600 nm. Each anthocyanin was quantified by means of a standard curve prepared by analysis of known amounts of the purified component under the same HPLC conditions.

RESULTS

Concentration of BCA. Anthocyanins in a commercially available black currant juice concentrate (65 °Brix, Iprona A.G.) were adsorbed on a column of cation-exchange resin (Amberlite 200CT) under acidic conditions (pH 1.4) and eluted with a solution of 25% (v/v) EtOH in 0.2 N hydrochloric acid to yield a BCA concentrate. HPLC analysis of the commercial juice and the BCA concentrate resulted in similar profiles, each showing four peaks when monitored at 520 nm, as shown in Figure 1A,B. The total anthocyanin contents of the former and the latter were 0.71 and 10.8%, respectively, indicating that the anthocyanins were concentrated 15-fold by this process in which the solid matter was decreased to about a fourth of the original amount (from 650 to 152 g).

Treatment of BCA Concentrate with β-Glucosidase or Hesperidinase. When the BCA concentrate was treated with β-glucosidase in acetate buffer (pH 3.5) at 40 °C, D3G and C3G were selectively hydrolyzed and the content of each was found to decrease as the enzyme reaction progressed as shown in Figure 2A. The HPLC profile after 8 h is shown in Figure 1C. As shown in Table 1, the contents of the hydrolyzed components, D3G and C3G, decreased to 0.08% and an undetectable level. On the other hand, D3R and C3R were unhydrolyzed and the contents remained at 4.89 and 3.63%, respectively. Regarding the recovery yields of the four components, the amounts of D3R and C3R were found to remain above 96% of the initial levels.

Treatment of the BCA concentrate (40 g) with hesperidinase under similar conditions produced a different result. The amounts of D3G and C3G increased to levels greater than the initial levels as the enzyme reaction progressed with an accompanying decrease in the amounts of D3R and C3R, as shown in Figure 2B. After

Table 1. Amounts, Contents, and Recovery Yields of the Individual Anthocyanins in the Isolation Process[a]

materials and preparations, weight as solid matter	anthocyanin: amount,[b] g (content,[c] %), and [recovery yield,[d] %]				
	D3R	D3G	C3R	C3G	sum of four components
1. commercial juice, 1710 g	5.81 (0.34) [286]	1.37 (0.08) [232]	4.45 (0.26) [297]	0.51 (0.03) [255]	12.14 (0.71) [281]
2. BCA concentrate, 40 g	2.03 (5.08) [100]	0.59 (1.48) [100]	1.50 (3.75) [100]	0.20 (0.50) [100]	4.32 (10.8) [100]
3. β-Glc-ase process					
a. after enzyme treatment	1.95 (4.89) [96.6]	0.03 (0.08) [5.1]	1.45 (3.63) [96.7]	nd	3.43 (8.60) [79.4]
b. after ODS separation	1.15 (>99.5) [56.7]	--	1.01 (>99.5) [67.3]	--	2.16 (>99.5) [50.0]
c. recrystallized	0.97 (>99.5%) [47.8]	--	0.76 (>99.5) [50.7]	--	1.73 (>99.5) [40.0]
4. Hes-ase process					
a. after enzyme treatment	0.02 (0.05) [1.0]	2.21 (5.54) [375]	nd	1.41 (3.52) [705]	3.64 (9.10) [84.3]
b. after ODS separation	−	1.09 (>99.5) [185]	--	0.84 (>99.5) [420]	1.93 (>99.5) [44.7]
c. recrystallized	--	0.90 (>99.5) [153]	--	0.62 (>99.5) [310]	1.52 (>99.5) [35.2]

[a] Abbreviations: β-Glc-ase, β-glucosidase; Hes-ase, hesperidinase; nd, not detected; −, not performed. [b] Expressed as the weight (g) of dry matter obtained from a starting BCA extract (40 g). [c] Expressed as the ratio % (w/w) of dry matter in each line. [d] Ratio of each figure to respective figure of BCA extract [100].

Table 2. Physical and Spectral Data for Four Components of Black Currant Anthocyanin[a]

	D3R·Cl		D3G·Cl		C3R·Cl		C3G·Cl	
mp, °C	>200		>200		>200		>200	
TG and DTG, loss % (°C)[b]								
from 30 to 150 °C	3.6 (97)		0.8 (87)		1.9 (112)		1.4 (89)	
from 150 to 300 °C	25.3 (224)		24.1 (258)		25.3 (214, 226)		22.3 (245)	
crystalline water, mol[e]	1.33		0.23		0.69		0.39	
molecular formula[d]	$C_{27}H_{31}ClO_{16}\cdot1.5H_2O$		$C_{21}H_{21}ClO_{12}\cdot0.5H_2O$		$C_{27}H_{31}ClO_{15}\cdot0.5H_2O$		$C_{21}H_{21}ClO_{11}\cdot0.5H_2O$	
UV, λ max (ϵ)[e]	520 (27800)		517 (27500)		512 (27400)		510 (26300)	
FAB-MS, m/z	611 $(C_{27}H_{31}O_{16})^+$		465 $(C_{21}H_{21}O_{12})^+$		595 $(C_{27}H_{31}O_{15})^+$		449 $(C_{21}H_{21}O_{11})^+$	
^{13}C and ^1H NMR[f]								
C2	160.0		158.3		161.1		159.8	
C3	144.1		144.1		144.1		144.1	
C4	133.9	8.32 s	132.7	8.10 s	134.7	8.40 s	133.9	8.32 s
C5	157.3		157.1		157.4		157.3	
C6	103.1	6.46 d	103.0	6.31 d	103.3	6.50 d	103.2	6.38 d
C7	168.9		168.7		168.9		168.7	
C8	95.5	6.46 d	95.1	6.20 d	95.6	6.54 d	95.3	6.38 d
C9	155.7		155.0		155.8		155.3	
C10	112.3		111.9		112.3		112.0	
C1′	118.4		117.8		119.8		119.3	
C2′	111.7	7.06 s	111.3	6.74 s	117.8	7.43 d	117.2	7.17 d
C3′	145.8		145.5		145.5		145.3	
C4′	143.3		143.3		154.2		154.1	
C5′	145.8		145.5		117.2	6.68 d	117.1	6.49 d
C6′	111.7	7.06 s	111.3	6.74 s	127.5	7.66 d	127.5	7.49 d
G1	101.9	5.20 d (7.3)	101.8	5.10 d (6.8)	102.1	5.16 d (7.3)	102.1	5.17 d (7.1)
G2	73.4	3.7[g] dd	73.3	3.7[g] dd	73.5	3.7[g] dd	73.5	3.7[g] dd
G3	76.6	3.7[g] dd	77.6	3.7[g] dd	76.6	3.7[g] dd	77.5	3.7[g] dd
G4	70.0	3.6[g] dd	70.2	3.6[g] dd	70.0	3.6[g] dd	70.1	3.6[g] dd
G5	76.4	3.79 m	76.9	3.7[g] m	76.3	3.77 m	76.9	3.7[g] m
G6	67.1	3.7[g] dd, 4.12 dd	61.6	3.92 dd, 4.10 dd	67.2	3.7[g] dd, 4.1[g] dd	61.5	3.92 dd, 4.07 dd
R1	101.2	4.8[g] d (1.5)			101.2	4.8[g] d (1.6)		
R2	70.7	3.91 dd			70.8	3.92 dd		
R3	71.1	3.66 dd			71.2	3.7[g] dd		
R4	72.7	3.35 dd			72.7	3.35 dd		
R5	69.4	3.6[g] dd			69.4	3.6[g] dd		
R6	17.2	1.10 d			17.3	1.10 d		

[a] Abbreviations: D3R, delphinidin 3-*O*-β-rutinoside, D3G, delphinidin 3-*O*-β-glucoside; C3R, cyanidin 3-*O*-β-rutinoside; C3G, cyanidin 3-*O*-β-glucoside; H and C, hydrogen and carbon of anthocyanin aglycon moiety; G, glucose moiety; R, rhamnose moiety. [b] Express weight loss % (at temperature). [c] Calculated by assuming the weight loss from 30 to 150 °C as crystal water. [d] Tentatively determined from the crystalline water and used for the calculation of e values in UV spectra. [e] Measured in 0.1 N HCl. [f] δ (J, Hz). [g] Chemical shift from 2D.

the enzyme reaction had progressed for 14 h, the HPLC profile (Figure 1D) showed that the D3R and C3R peaks both had almost disappeared and the mixture consisted of two major components, D3G and C3G. As shown in Table 1, the recovery yields of D3G and C3G were found to increase to 375% (from 0.59 to 2.21 g) and 705% (from 0.20 to 1.41 g) compared to the starting amounts.

Isolation of Anthocyanin Components. Each mixture treated with two kinds of enzymes was applied to a column of Amberlite XAD-7HP, and the adsorbed anthocyanins were eluted with aqueous MeOH (MeOH/H_2O = 80:20) containing 0.1% (v/v) TFA. The eluate in each instance was concentrated and applied to a preparative HPLC with an ODS 120T (∅ 5.5 cm × 30 cm) column. Two predominant components in the eluate in each instance were separated under the conditions described in Materials and Methods as single peaks by HPLC. Fine colored crystals of the four components, D3R, D3G, C3R, and C3G, were obtained as the flavylium chloride by recrystallization from methanol/5.0% hydrochloric acid (25:2), and the recovery yields from the BCA concentrate were calculated to be 47.8, 153, 50.7, and 310%, respectively. The purity was >99.5% in each case.

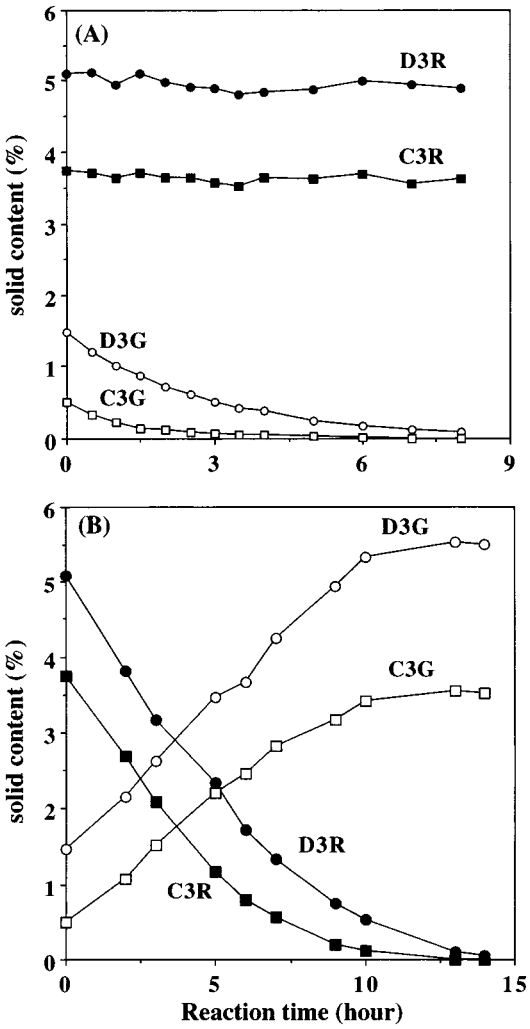

Figure 2. Time course of changes in the amounts of BCA upon treatment with β-glucosidase (A) and hesperidinase (B): D3R (●); C3R (■); D3G (○); C3G (□).

Characterization of the Anthocyanins. Structural characterization of the four components was performed, and their physical and spectral properties are shown in Table 2. The UV and FAB-MS spectral properties of D3R, C3R, D3G, and C3G were in good agreement with those reported by Degenhardt et al. (*13*). The ^{13}C and ^{1}H NMR spectra of D3G and C3G were consistent with data in the literature (*3*). The NMR spectra of D3R and C3R were in good agreement with those of delphinidin 3-*O*-β-rutinoside and cyanidin 3-*O*-β-rutinoside, as α-rhamnosyl anomeric protons (R1) were detected that had characteristic coupling constants of 1.5 Hz (d, δ 4.8) and 1.6 Hz (d, δ 4.8), respectively (*14*). The structures of the four components are shown in Figure 3. The thermal properties of the four crystals were analyzed by TG-DTG, and the curves showed a small weight loss, 0.8−3.6%, at 87−112 °C and a subsequent dramatic weight loss, 22.3−25.3%, at 214−258 °C. As the loss in the first stage was thought to be due to loss of crystalline water, the flavylium chlorides were calculated to have a crystal water content ranging from 0.23 to 1.33 molecules.

For quantitative analysis, standard curves of the purified components were prepared, and the amount, content, and recovery yield of each of the four anthocyanin components were calculated at each step of the isolation process, as shown in Table 1.

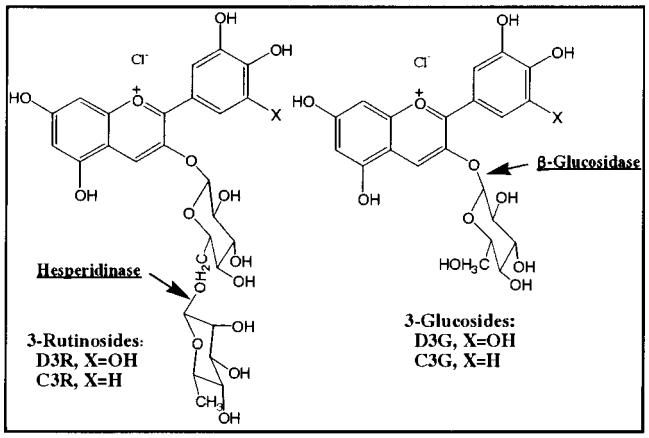

Figure 3. Chemical structures of BCA, 3-rutinosides, and 3-glucosides. Arrows show the bonds subject to regioselective cleavage by hesperidinase and β-glucosidase.

DISCUSSION

A few anthocyanins, such as C3G and peralgonidin 3-glucoside in red beans, have been isolated by preparative HPLC (*3*), but application of this method for the purification of BCA components was found to have a limitation in that the anthocyanin peaks were not adequately separated in the preparative-scale operation. Four components of BCA were reported to be preparatively separated by centrifugal partition chromatography (*12*) and high-speed countercurrent chromatography (*13*), but further improvement of these methods seems to be needed for good separation because overloading of the sample resulted in overlapping of adjacent components and a decrease in the recovery yields.

Reduction of the number of components of BCA was considered to provide an advantage to obtain well-resolved peaks and to make baseline separation possible even when a large amount of sample was injected. Judging from the HPLC profile shown in Figure 1B, selective removal of either glucosides or rutinosides appeared to be helpful to obtain well-resolved peaks. Enzymatic transformation of the flavonoid glycosides in fruits juices has been used for a few purposes, such as for improvement of clarification using a pectinolytic enzyme (*15*) and for formation of aroma (*16*). In the latter case, hesperidinase was reported to have high catalytic efficiency in the hydrolysis of α-rhamnosides having a $\alpha(1-6)$linkage, such as hesperidine and linalyl β-rutinoside. However, there has been no report of the regioselective hydrolysis of a similar $\alpha(1\rightarrow6)$linkage of anthocyanin 3-*O*-β-rutinosyl moieties as yet.

By treatment of BCA with β-glucosidase from almond, the amounts of the two small constituents, D3G and C3G, diminished to almost undetectable levels, whereas the amounts of the two larger constituents, D3R and C3R, were found to remain unchanged, as shown in Figure 2A. These findings support the view that both glucosides were hydrolyzed to aglycons, which are easily decomposed to undetectable compounds under the enzyme reaction conditions employed (at pH 3.5) (*17*). As the amounts of D3R and C3R remained >95% of the initial levels without any structural change, the β-glucosidase was confirmed to act only on the terminal 3-*O*-β-glucosides of D3G and C3G, showing no activity in hydrolysis of the internal 3-*O*-β-glucosyl bond of the rutinosyl moiety, as shown in Figure 3. In the case of hesperidinase treatment, as shown in Figure 2B, the peak areas of D3G and C3G increased as those of two

the larger components, D3R and C3R, decreased during the 15 h reaction period. This increase can be explained in terms of the expected conversion of the rutinosides into the glucosides, involving removal of the terminal α-rhamnosyl moieties from D3R and C3R. This is catalyzed by α-rhamnosidase activity in the hesperidinase preparation, and the cleavage position in the rutinosyl moieties is shown by an arrow in Figure 3. After the enzyme reaction, the amounts of D3G and C3G had increased to 375 and 705% of the initial amounts, and this result showed that the regioselectivity was so high that both rutinosides were converted into the glycosides in quantitative yields.

As shown in Figure 2C,D, these enzyme treatments reduced the number of components of BCA from four to two, and consequently the spaces between adjacent peaks in the HPLC profile were made larger than those before the enzyme treatment. In each instance the enzyme-treated BCA was applied to an Amberlite XAD-7HP column followed by elution with aqueous MeOH and, thereafter, by preparative HPLC separation with an ODS 120T column the four components D3R, C3R, D3G, and C3G were obtained in high purity and with good recovery yields. As shown in Table 1, all four components were isolated as fine crystals of flavylium chloride at >99.5% purity and the recovery yields of D3R, D3G, C3R, and C3G were 47.8, 153, 50.7, and 310%, respectively, based on the content of each component in the BCA concentrate.

Using these crystals, structural identification was performed, and their physical and spectral properties were determined as shown in Table 2. UV, FAB-MS, and ^{13}C and ^{1}H NMR spectral data were consistent with the assigned structures, and the TG-DTG curves suggested that all of the crystals had crystal water, calculated to be in the range of 0.23−1.33 molecules. The crystals were too fine to perform X-ray crystallography. The crystal water contents of D3R, D3G, C3R, and C3G were tentatively estimated to be 1.5, 0.5, 0.5, and 0.5 molecules, respectively, and these molecular formula were used for calculation of the ϵ values in UV analysis and in quantitative analysis of the BCA components by HPLC. Continued attempts to prepare crystals suitable for X-ray crystallography for determination of the exact crystal structures are in progress.

Not only are the purified anthocyanins described in this paper useful as standard samples for quantitative measurement, they will also be useful for the evaluation of their physiological activities.

ABBREVIATIONS USED

BCA, black currant anthocyanins; C3G, cyanidin 3-*O*-*β*-glucoside; C3R, cyanidin 3-*O*-*β*-rutinoside; D3G, delphinidin 3-*O*-*β*-glucoside; D3R, delphinidin 3-*O*-*β*-rutinoside; FAB-MS, fast atom bombardment mass spectra; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; MeOH, methanol; ODS, octadecyl silica gel.

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