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Anthocyanins from New Red Leaf Tea 'Sunrouge'

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ABSTRACT: New red leaf tea cultivar 'Sunrouge' (*Camellia taliensis* × *Camellia sinensis*), for which an application for registration was made in 2009, is an anthocyanin-rich tea. The anthocyanin content of 'Sunrouge' was the highest among 4 tea cultivars, and was 8.4 times higher than that of 'Yabukita'. We purified and isolated 6 anthocyanins from 'Sunrouge' by chromatography, and identified them by LC/MS/MS and NMR analysis. As a result, the four anthocyanins were identified as delphinidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl)galactopyranoside (**2**), delphinidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl)glucopyranoside (**3**), cyanidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl)galactopyranoside (**4**), and cyanidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl)glucopyranoside (**5**), and the other two were estimated as delphinidin-(*Z*)-*p*-coumaroylgalactopyranoside (**1**), petunidin-(*E*)-*p*-coumaroylgalactopyranoside (**6**). Compound **3** was found in tea for the first time. In general, anthocyanins have various bioactivities, including relieving eyestrain and antioxidative effects, so it is expected that drinking 'Sunrouge' tea brings in similar bioactivities.

KEYWORDS: anthocyanin, tea (*Camellia sinensis*), 'Sunrouge' (*Camellia taliensis* × *Camellia sinensis*)

INTRODUCTION

Tea (*Camellia sinensis*) is consumed all over the world, especially in Japan and China. It is reported that green tea has various bioactivities, including antioxidative,[1] antimutagenic and anticarcinogenic,[2] antihypertensive, oral health-promoting, solar ultraviolet-protective, glucose tolerance and insulin sensitivity-related, antibacterial, antiviral,[3] and body weight-reducing effects.[4]

Camellia taliensis is closely related to *C. sinensis* and is drunk as 'Dalicha' in China. An anthocyanin-rich tea cultivar 'Cha Chuukanbohon Nou 6' (*Camellia taliensis* × *Camellia sinensis*) was bred from seedling of *Camellia taliensis* by the National Institute of Vegetable and Tea Sciences. Anthocyanins have various bioactivities, including antioxidative,[5] anti-inflammatory,[6] and eyestrain-relieving activities.[7] Drinking anthocyanin-rich tea enables simultaneous ingestion of catechins, which are functional components of tea, and anthocyanins, but it is difficult to cultivate 'Cha Chuukanbohon Nou 6' since it has a small number of buds.

Therefore, breeding easily cultivated and anthocyanin-rich tea was carried out, and new red leaf tea cultivar 'Sunrouge' (*Camellia taliensis* × *Camellia sinensis*) was selected from 'Cha Chuukanbohon Nou 6' natural crossing, for which an application for registration was made in 2009. In this study, we quantified the total anthocyanin contents of 4 tea cultivars: 'Sunrouge', 'Cha Chuukanbohon Nou 6', 'Benibana-cha' (*C. sinensis*), the anthocyanin-rich tea cultivar, and 'Yabukita' (*C. sinensis*), the common tea cultivar in Japan. Furthermore, we isolated and identified 6 unknown anthocyanins, which were detected by the HPLC analysis of 'Sunrouge' extract.

MATERIALS AND METHODS

Chemical and Materials. Solvents were of special grade unless otherwise stated. Ethanol (EtOH), acetic acid, ethyl acetate, acetonitrile, trifluoroacetic acid (TFA), formic acid, hydrochloric acid (HCl aq), and deuterium oxide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), HPLC-grade acetonitrile and methanol-d_4 were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and TFA-*d* was purchased from SIGMA-Aldrich Inc. (St. Louis, MO, USA). HPLC-grade cyanidin-3-*O*-*β*-glucoside was purchased from Extrasynthese Co. (Lyon, France). Amberlite XAD-7HP was purchased from Organo Co., Ltd. (Tokyo, Japan), and Inertsil ODS-3 column (20 × 250 mm, 4 *μ*m; 4.6 × 250 mm, 4 *μ*m; 2.0 × 150 mm, 3 *μ*m) and Inertsil PH-3 column (20 × 250 mm, 5 *μ*m) were from GL Sciences, Inc., Japan (Tokyo, Japan). 'Sunrouge' tea leaves were obtained from the National Institute of Vegetable and Tea Sciences.

Quantifications of Anthocyanins. Anthocyanins of 'Sunrouge', 'Cha Chuukanbohon Nou 6', 'Benibana-cha', and 'Yabukita' were quantified equivalent to cyanidin-3-*O*-*β*-glucoside concentration by the following method. Absorbance of 15% acetic acid solutions, prepared in the range of 0.781−50 *μ*M of cyanidin-3-*O*-*β*-glucoside, was measured, and a calibration curve was created. Then, absorbance of 15% acetic acid extracts of four tea cultivar leaves (n = 18, except for 'Benibana-cha', n = 12) was measured, and anthocyanin contents were calculated from this calibration curve.

Extraction and Isolation of Anthocyanin from 'Sunrouge'. 'Sunrouge' tea leaves (87.2 g) were extracted with 15% acetic acid (600 mL × 3), and additionally extracted with 15% acetic acid-containing

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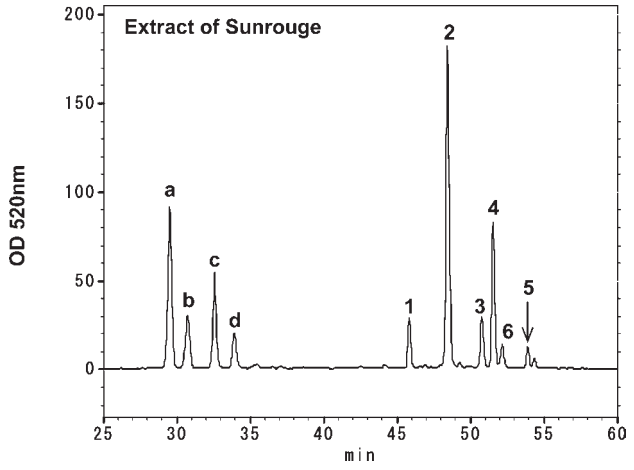

Figure 1. HPLC chromatogram of an extract of 'Sunrouge'. Anthocyanins a−d were identified by HPLC analysis; a, delphinidin-3-*O*-*β*-D-galactopyranoside; b, delphinidin-3-*O*-*β*-D-glucopyranoside; c, cyanidin-3-*O*-*β*-D-galactopyranoside; d, cyanidin-3-*O*-*β*-D-glucopyranoside.

50% EtOH (600 mL × 4). The combined extract was evaporated and freeze-dried. The freeze-dried solid extract was washed with ethyl acetate (400 mL × 2) and then evaporated to remove the ethyl acetate. This extracted solid was dissolved in 1% acetic acid and subjected to XAD-7HP column (45 × 400 mm) chromatography by stepwise elution with 1% acetic acid (2500 mL), 1% acetic acid-containing 30% EtOH (2500 mL), 1% acetic acid-containing 50% EtOH (2500 mL), and 1% acetic acid-containing 70% EtOH (2500 mL). HPLC analysis of each fraction showed that 1% acetic acid-containing 50% EtOH fraction had 6 unknown anthocyanins (compound **1**−**6**, Figure 1). This fraction was subjected to preparative HPLC using an Inertsil ODS-3 column (20 × 250 mm, 4 *μ*m). The mobile phase A was 0.1% TFA, and the mobile phase B was 0.1% TFA-containing 50% acetonitrile. 38% B isocratic elution gave 6 anthocyanin fractions. Then, each fraction was repeatedly subjected to reverse-phase HPLC using Inertsil ODS-3 column (20 × 250 mm, 4 *μ*m) and Inertsil PH-3 column (20 × 250 mm, 5 *μ*m) to obtain the 6 anthocyanins. The mobile phase A was 0.1% TFA, and the mobile phase B was 0.1% TFA-containing 50% acetonitrile; or the mobile phase A was 0.1% HCl aq, and the mobile phase B was 0.1% HCl aq-containing 50% acetonitrile. The detection wavelength was 520 nm and flow rate was 18.9 mL/min in all these analyses.

HPLC. The measuring of the 'Sunrouge' extract was carried out with the HPLC system, with an L-2130 pump, an L-2300 column oven, an L-2200 autosampler, and an L-2450 DAD detector (Hitachi Ltd., Tokyo, Japan). The 'Sunrouge' extract was subjected to reverse-phase HPLC using an Inertsil ODS-3 column (4.6 × 250 mm, 4 *μ*m). The flow rate was 1.0 mL/min. The mobile phase A was 0.1% TFA, and the mobile phase B was 0.1% TFA-containing 50% acetonitrile. The gradient conditions were as follows: 0−60 min, 5−60% B; 60−70 min, 60−100% B. The purification and isolation of compounds were conducted using the HPLC system, with an L-7150 pump, an L-7420 UV−vis detector (Hitachi, Ltd., Tokyo, Japan), a column oven 505 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and a manual sample injector Rheodyne 7725i (Rheodyne, Cotati, CA, USA). The flow rate was 18.9 mL/min. The LC/MS/MS measuring of the 'Sunrouge' anthocyanins was carried out using the HPLC system, with an L-2100 pump, an L-2300 column oven, an L-2200 autosampler, and an L-2400 UV detector (Hitachi Ltd., Tokyo, Japan). Each anthocyanin was subjected to reverse-phase HPLC using an Inertsil ODS-3 column (2.0 × 150 mm, 3 *μ*m). The flow rate was 0.2 mL/min. The mobile phase A was 0.1% formic acid, and the mobile phase B was 0.1% formic acid-containing 50% acetonitrile. The gradient conditions were as follows: 0−60 min, 5−60% B; 60−70 min, 60−100% B. The detection wavelength was 520 nm in all these analyses.

Table 1. Anthocyanin Contents of 4 Tea Cultivars[a]

tea cultivar	anthocyanin content (%)	SD
'Sunrouge'	0.211	0.047
'Cha Chuukanbohon Nou 6'	0.149	0.032
'Benibana-cha'	0.135	0.011
'Yabukita'	0.025	0.008

[a] Data represent the mean of n = 18, except for Benibana-cha, n = 12.

MS/MS. The LC/MS/MS measuring of the tea leaf anthocyanins was carried out with a QSTAR system (Applied Biosystems Inc., California, USA). The measuring was carried out by the following method: electro ion spray mode; ion spray voltage, 5500 V; temperature, 450 °C; curtain gas, 30 (arbitrary units); ion source gas, 50 (arbitrary units); automatic collision energy mode.

NMR. ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), heteronuclear single-quantum coherence (HSQC), correlation spectroscopy (COSY), and heteronuclear multiple-bond connectivity (HMBC) spectra were recorded in MeOH-d_4−TFA-d (9:1) with a Bruker AV600 (Bruker Biospin GmbH, Rheinstetten, Germany).

RESULTS AND DISCUSSION

Anthocyanin Contents. The anthocyanin contents of 4 tea cultivars (first crop) are shown in Table 1. The anthocyanin content of 'Sunrouge' was the highest in 4 tea cultivars, and 1.4, 1.7, and 8.4 times higher than those of 'Cha Chuukanbohon Nou 6', 'Benibana-cha', which is anthocyanin-rich, and 'Yabukita'. Therefore, 'Sunrouge' is a remarkably anthocyanin-rich tea cultivar.

Identification. The MS/MS analysis of compounds **1**, **2**, and **3** showed a molecular ion at m/z 611.1409, 611.1444, and 611.1451 (the exact mass calculated for $C_{30}H_{27}O_{14}^+$ was m/z 611.13953), and a major fragment ion at m/z 303.0535, 303.0555, and 303.0557, respectively, corresponding to the aglycon delphinidin (the exact mass calculated for $C_{15}H_{11}O_7^+$ was m/z 303.0505). Compounds **1** and **2** were (*E*)−(*Z*) isomers because they were not separated completely from each other.[8] Therefore, compound **1** was estimated as delphinidin-(*Z*)-*p*-coumaroylgalactopyranoside. ^{1}H NMR analysis showed that compounds **2** and **3** had a (*E*)-*p*-coumaroyl moiety ($J_{\alpha-\beta}$ = 15.9 Hz), compound **2** had a *β*-galactose moiety (J = 7.8 Hz)[9] and compound **3** had a *β*-glucose moiety (J = 7.6 Hz),[10] and the positions of linkage between delphinidin and hexose, and between hexose and coumaroyl, were determined by HMBC (Table 2). Therefore compound **2** was identified as delphinidin-3-*O*-*β*-D-(6-(*E*)-coumaroyl)galactopyranoside and compound **3** was identified as delphinidin-3-*O*-*β*-D-(6-(*E*)-coumaroyl)glucopyranoside (Figure 2).

The MS/MS analysis of compounds **4** and **5** showed a molecular ion at m/z 595.1411, 595.1489 (the exact mass calculated for $C_{30}H_{27}O_{13}^+$ was m/z 595.14462), and a major fragment ion at m/z 287.0612, 287.0608 corresponding to the aglycon cyanidin (the exact mass calculated for $C_{15}H_{11}O_6^+$ was m/z 287.0556). ^{1}H NMR analysis showed that they had an (*E*)-*p*-coumaroyl moiety ($J_{\alpha-\beta}$ = 15.9 Hz), compound **4** had a *β*-galactose moiety (J = 7.6 Hz)[11] and compound **5** had a *β*-glucose moiety (J = 7.7 Hz),[12] and the positions of linkage between delphinidin and hexose, and between hexose and coumaroyl, were determined by HMBC (Table 2). So compound **4** was

Table 2. ^{1}H NMR Data for Compounds 2, 3, 4, and 5 (in 9:1 CD_3OD/TFA-*d*)

	2	3	4	5
aglycon				
	delphinidin	delphinidin	cyanidin	cyanidin
4	8.89 s	8.88 s	8.95 s	8.92 s
6	6.54 s	6.52 s	6.85s	6.83 s
8	6.76 s	6.76 s	6.55 s	6.55 s
2′	7.77 s	7.72 s	8.08 d (2.3)	8.03 d (2.3)
5′			7.05 d (8.8)	7.03 d (8.6)
6′	7.77 s	7.72 s	8.26 dd (8.7, 2.2)	8.24 dd (8.7, 2.3)
sugar				
	galactosyl	glucosyl	galactosyl	glucosyl
1	5.30 d (7.8)	5.33 d (7.6)	5.30 d (7.6)	5.33 d (7.7)
2	4.01 brd (3.2)	3.76 t (8.3)	4.01 brd (2.6)	3.73 t (8.3)
3	3.75 dd (9.7, 3.3)	3.60 t (9.0)	3.74 dd (9.6, 3.4)	3.59 t (9.0)
4	4.06 dd (9.6, 7.8)	3.48 t (9.1)	4.05 dd (9.5, 7.8)	3.48 t (9.4)
5	4.12 dd (8.8, 4.1)	3.85 td (8.5, 2.2)	4.13 dd (8.8, 3.0)	3.85 td (8.5, 2.2)
6a	4.30 dd (11.9, 3.9)	4.38 dd (11.9, 7.6)	4.31 dd (11.9,3.6)	4.38 dd (11.9, 2.2)
6b	4.59 dd (11.6, 8.9)	4.52 dd (11.9, 2.3)	4.59 dd (11.7, 9.0)	4.53 dd (11.9, 7.7)
p-coumaroyl				
2,6	7.28 d (8.5)	7.29 d (8.6)	7.26 d (8.5)	7.30 d (8.5)
3,5	6.77 d (8.5)	6.77 d (8.6)	6.80 d (8.5)	6.80 d (8.5)
α	6.23 d (15.9)	6.22 d (16.0)	6.25 d (15.9)	6.23 d (15.9)
β	7.44 d (15.9)	7.42 d (16.0)	7.45 d (15.9)	7.44 d (15.9)

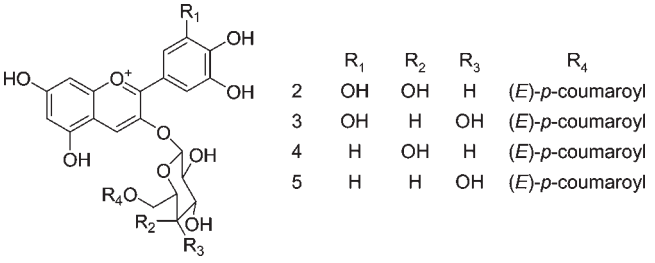

Figure 2. Structures of anthocyanins isolated from 'Sunrouge'.

identified as cyanidin-3-*O*-*β*-D-(6-(*E*)-coumaroyl)galactopyranoside and compound **5** was identified as cyanidin-3-*O*-*β*-D-(6-(*E*)-coumaroyl)glucopyranoside.

The MS/MS analysis of compound **6** showed a molecular ion at *m*/*z* 625.1624 (the exact mass calculated for $C_{31}H_{29}O_{14}^+$ was *m*/*z* 625.1557) and a major fragment ion at *m*/*z* 317.0713. The ^{1}H NMR data was completely assigned, but HMBC correlation between 3′-C and -OMe protons corresponded to the aglycon petunidin (the exact mass calculated for $C_{16}H_{13}O_7^+$ was *m*/*z* 317.0661).[13] ^{1}H NMR analysis showed that **6** has an (*E*)-coumaroyl moiety and a galactose moiety.[9] Then, compound **6** was estimated as petunidin-(*E*)-*p*-coumaroylgalactopyranoside.

Compound **3** was found in tea for the first time, although compounds **2**, **4** and **5** were previously reported for the genus *Camellia*.[9,11,12] Similarly, compound **6** with a petunidin moiety was found in tea for the first time. Moreover, four known anthocyanins a−d, identified respectively as delphinidin-3-*O*-*β*-D-galactopyranoside, delphinidin-3-*O*-*β*-D-glucopyranoside, cyanidin-3-*O*-*β*-D-galactopyranoside, and cyanidin-3-*O*-*β*-D- glucopyranoside, were observed in Figure 1. Additionally, Figure 1 shows that the peak intensities of compounds **2** and **4** were large and both have galactose as a hexose, which suggested that delphinidin or cyanidin mainly bound to galactose not glucose in the biosynthesis pathway of Sunrouge. It was reported that biosynthesis of anthocyanin-glucoside or -galactoside differed between plant species; for example, glucoside was larger in black soybean (*Glycine max*) and galactoside was larger in apple (*Malus domestica*). The anthocyanin biosynthesis depends on the major anthocyanin biosynthesis enzymes, UDP-glucose:flavonoid 3-*O*-glucosyl-transferase,[14,15] or UDP-galactose:flavonoid 3-*O*-galactosyltransferase and UDP-glucose 4-epimerase,[16] and it is predicted that the major enzyme of 'Sunrouge' is the latter. Furthermore, anthocyanin with petunidin-bound glucose was not detected in this study, but there is a possibility that 'Sunrouge' has a minor compound with glucose substituted for galactose in the structure of compound **6**.

In general, anthocyanins have various bioactivities, including relieving eyestrain and antioxidative effects, so it is expected that drinking 'Sunrouge' tea brings in similar bioactivities.

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ABBREVIATIONS USED

LC/MS/MS, liquid chromatography tandem mass spectrometry; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography

REFERENCES

(1) Anesini, C.; Ferraro, G. E..; Filip, R. Total polyphenol content and antioxidant capacity of commercially available tea (*Camellia sinensis*) in Argentina. *J. Agric. Food Chem.* **2008**, *56*, 9225–9229.

(2) Friedman, M.; Mackey, B. E.; Kim, H.; Lee, I.; Lee, K.; Lee, S.; Kozukue, E.; Kozukue, N. Structure-activity relationships of tea compounds against human cancer cells. *J. Agric. Food Chem.* **2007**, *55*, 243–253.

(3) Cabrera, C.; Artacho, R.; Giménez, R. Beneficial effects of green tea—a review. *J. Am. Coll. Nutr.* **2006**, *25*, 79–99.

(4) Phung, O. J; Baker, W. L.; Matthews, L. J.; Lanosa, M.; Thorne, A.; Coleman, C. I. Effect of green tea catechins with or without caffeine on anthropometric measures: a systematic review and meta-analysis. *Am. J. Clin. Nutr.* **2010**, *91*, 73–81.

(5) Faria, A.; Oliveira, J.; Neves, P.; Gameiro, P.; Santos-Buelga, C.; Freitas, V.; Mateus, N. Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *J. Agric. Food Chem.* **2005**, *53*, 6896–6902.

(6) Kowalczyk, E.; Krzesiński, P.; Kura, M.; Szmigiel, B.; Blaszczyk., J. Anthocyanins in medicine. *Pol. J. Pharmacol.* **2003**, *55*, 699–702.

(7) Nakaishi, H.; Matsumoto, H.; Tominaga, S.; Hirayama, M. Effects of black currant anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alteration in healthy humans. *Altern. Med. Rev.* **2000**, *5*, 553–562.

(8) Yoshida, K.; Kondo, T.; Kameda, K.; Goto, T. Structure of anthocyanins isolated from purple leaves of *Perilla ocimoides* L. var. *crispa* Benth and their isomerization by irradiation of light. *Agric. Biol. Chem.* **1990**, *54*, 1745–1751.

(9) Terahara., N.; Takeda, Y.; Nesumi, A.; Honda, T. Anthocyanins from red flower tea (Benibana-cha), *Camellia sinensis*. *Phytochemistry* **2001**, *56*, 359–361.

(10) Mori, M.; Kondo, T.; Yoshida, K. Cyanosalvianin, a supramolecular blue metalloanthocyanin, from petals of *Salvia uliginosa*. *Phytochemistry* **2008**, *69*, 3151–3158.

(11) Li, J. B.; Hashimoto, F.; Shimizu, K.; Sakata, Y. A new acylated anthocyanin from the red flowers of *Camellia hongkongensis* and characterization of anthocyanins in the section *Camellia* species. *J. Integr. Plant Biol.* **2009**, *51*, 545–552.

(12) Li, J.; Hashimoto, F.; Shimizu, K.; Sakata, Y. Anthocyanins from red flowers of *Camellia reticulata* LINDL. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 2833–2836.

(13) Hosokawa, K.; Fukunaga, Y.; Fukushi, E.; Kawabata, J. Seven acylated anthocyanins in the blue flowers of *Hyacinthus orientalis*. *Phytochemistry* **1995**, *38*, 1293–1298.

(14) Lee, J. H.; Kang, N. S.; Shin, S.; Shin, S.; Lim, S.; Suh, D.; Baek, I.; Park, K.; Ha, T. J. Characterisation of anthocyanins in the black soybean (*Glycine max* L.) by HPLC-DAD-ESI/MS analysis. *Food Chem.* **2009**, *112*, 226–231.

(15) Kovinich, N.; Saleem, A.; Arnason, J. T.; Miki, B. Functional characterization of a UDP-glucose:flavonoid 3-*O*-glucosyltransferase from the seed coat of black soybean (*Glycine max* (L.) Merr.). *Phytochemistry* **2010**, *71*, 1253–1263.

(16) Ban, Y.; Kondo, S.; Ubi, B. E.; Honda, C.; Bessho, H.; Moriguchi, T. UDP-sugar biosynthetic pathway: contribution to cyanidin 3-galactoside biosynthesis in apple skin. *Planta* **2009**, *230*, 871–881.