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Rapid Isolation of Cyanidin 3-Glucoside and Peonidin 3-Glucoside from Black Rice (*Oryza sativa*) Using High-Performance Countercurrent Chromatography and Reversed-Phase Column Chromatography

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Abstract – Anthocyanins are water soluble plant pigments which are responsible for the blue, red, pink, violet colors in several plant organs such as flowers, fruits, leaves and roots. In recent years, anthocyanin-rich foods have been favored as dietary supplements and health care products due to diverse biological activities of anthocyanins including antioxidant, anti-allergic, anti-diabetic, anti-microbial, anti-cancer and preventing cardiovascular disease. High-performance countercurrent chromatography (HPCCC) coupled with reversed-phase medium pressure liquid chromatography (RP MPLC) method was applied for the rapid and efficient isolation of cyanidin 3-glucoside (C3G) and peonidin 3-glucoside (P3G) from black rice (*Oryza sativa* L., Poaceae). The crude black rice extract (500 mg) was subjected to HPCCC using two-phase solvent system composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.01% trifluoroacetic acid (TBME/B/A/0.01% TFA, 1 : 3 : 1 : 5, v/v, flow rate - 4.5 mL/min, reversed phase mode) to give enriched anthocyanin extract (37.4 mg), and enriched anthocyanin extract was sequentially chromatographed on RP-MPLC to yield C3G (16.5 mg) and P3G (8.7 mg). The recovery rate and purity of isolated C3G were 76.0% and 98.2%, respectively, and those of P3G were 58.3% and 96.3%, respectively. The present study indicates that HPCCC coupled with RP-MPLC method is more rapid and efficient than multi-step conventional column chromatography for the separation of anthocyanins.

Keywords – Black rice, High-performance countercurrent chromatography, Reversed-phase medium pressure liquid chromatography, Cyanidin 3-glucoside, Peonidin 3-glucoside

Introduction

Anthocyanins are water soluble plant pigments which are synthesized through shikimic acid and mevalonate pathways, and responsible for the blue, red, pink, violet colors in several plant organs such as flowers, fruits, leaves and roots.[1] In recent years, anthocyanin-rich foods have been favored as dietary supplements and health care products because a lot of studies have revealed that anthocyanins possess diverse biological activities including antioxidant, anti-allergic, anti-diabetic, anti-microbial, anti-cancer and preventing cardiovascular disease.[2] Anthocyanin researches have recently intensified because potential beneficial effects for human health are being discovered. Therefore, the demand for pure anthocyanins has grown over time for the evaluation of *in-vivo* screening and quality control of dietary supplements and functional foods.

Black rice (*Oryza sativa* L., Poaceae) is a special cultivar of rice and widely consumed in Eastern Asia regions such as Korea, China and Japan as health care food for containing plenty of anthocyanins. Many phytochemical investigations have reported that cyanidin 3-glucoside (C3G) and peonidin 3-glucoside (P3G) are the main anthocyanins of black rice.[3-5] Generally, the isolation of anthocyanins have been performed using multi-step conventional column chromatography which requires time and labor consuming isolation process, which is not suited for the unstable compounds, especially anthocyanins.

Countercurrent chromatography (CCC) is a liquid-liquid chromatography which uses only liquids for mobile and stationary phases, thus there is no chemical degrada-

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tion and irreversible adsorption onto stationary phase of target compounds occurring in solid support based column chromatography.[6] For the aforementioned reasons, CCC methods have been used to isolate varieties of natural products from medicinal plants.[7,8] In this study, we combined high-performance CCC (HPCCC) and reversed-phase medium pressure liquid chromatography (RP MPLC) for the rapid and efficient isolation of C3G and P3G from black rice.

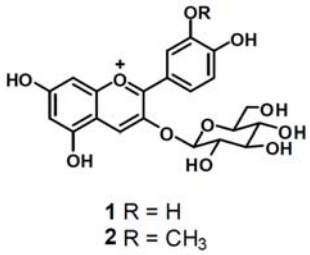

Fig. 1. Chemical structures of C3G (**1**) and P3G (**2**).

Experimental

General experimental procedure – The HPCCC instrument was a Spectrum HPCCC (Dynamic Extractions, Berkshire, UK) possessing two set of a semi-preparative coil of 70.5 mL with a 3.2-mm I.D. with β-Values ranged from 0.52 to 0.86. The Spectrum HPCCC was combined with a 2487 dual λ absorbance detector (Waters, MA, USA), a 1525 binary HPLC pump (Waters, MA, USA), a FC 204 fraction collector (Gilson, WI, USA) and a CCA-1111 circulatory temperature regulator (Eyela, Tokyo, Japan) to maintain the internal temperature at 30 ºC. Medium pressure liquid chromatography (MPLC) system comprised an IOTA S 300 pump (ECOM, Prague, Czech Republic), 2487 dual λ absorbance detector (Waters, MA, USA) and a Foxy® R2 fraction collector (Teledyne Isco, NE, USA). Reversed-phase (RP) column for MPLC was Redisep®Rf 130 g C18 Reversed Phase column (Teledyne Isco, NE, USA). HPLC analyses were conducted by an Alliance HPLC system (Waters, MA, USA) using an Eclipse XDB-C18 column (4.6 × 250 mm I.D., 5 μm, Agilent Technologies, CA, USA). organic solvents for HPCCC and MPLC were purchased from Daejung-Chemical and Metals Co. Ltd. (Kyunggi-Do, Korea) and deionized water was produced by Millipore Milli-Q water purification system (Millipore, USA). ^{1}H NMR spectra were recorded on a Bruker AscendTM 500 spectrometer (Bruker, Germany).

Preparation of sample material – Black rice (600 g) was purchased from the local food market in Buchoen-si, and extracted with methanol (acidified with 0.5% TFA, 2 L × 3 hr × three times) to give crude black rice extract (BRE, 38 g). The crude BRE was stored at −80 ºC prior to experiment.

HPLC condition – HPLC analysis was performed using an Eclipse XDB-C18 column (4.6 × 250 mm I.D., 5 μm, Agilent Technologies, CA, USA). The flow rate was 1.0 mL/min and the detection wavelength was 520 nm, and the sample temperature was maintained at 10 ºC. The organic mobile phase (A) was a methanol acidified 0.1% TFA, and the aqueous mobile phase (B) was a 0.1% TFA. The mobile phase gradient was 10% - 40% A (0 - 20 min). The samples were injected with the volume of 20.0 μL. The concentrations of sample were 10 mg/mL (BRE), 1 mg/mL (enriched anthocyanin extract, C3G and P3G), respectively.

HPCCC and RP-MPLC procedure – The BRE (500 mg) was dissolved in a 1 : 1 mixture of the upper and lower phases of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/0.01% trifluoroacetic acid (TBME/B/A/0.01% TFA, 1 : 3 : 1 : 5, v/v) and subjected to HPCCC to give enriched-anthocyanin extract (37.4 mg). The HPCCC parameters are as follows: stationary and mobile phase - upper phase and lower phase of TBME/B/A/0.01% TFA, 1 : 3 : 1 : 5, v/v, respectively; flow rate - 4.5 mL/min; rotational speed - 1600 rpm; detection wavelength - 520 nm). The enriched anthocyanin extract (37.4 mg) obtained from HPCCC was subjected to RP-MPLC. The mobile phases was methanol-water (30 : 70, v/v) acidified by 0.1% TFA. The flow rate was 20 mL/min and detection wavelength was 520 nm.

Cyanidin 3-glucoside (C3G): Q-TOF/MS: *m/z* 449.1094 $[M]^+$ (calcd 449.1078 for $C_{21}H_{21}O_{11}^+$), 287.2558 $[M\text{-}Glc]^+$; ^{1}H NMR [500 MHz, DMSO-d_6-TFA-d_1 (9 : 1, v/v)] δ 8.89 (1H, s, H-4), 8.23 (1H, dd, J = 8.8, 2.2 Hz, H-6'), 8.01 (1H, d, J = 2.3 Hz, H- 2'), 7.03 (1H, d, J = 8.7 Hz, H-5'), 6.90 (1H, d, J = 2.0 Hz, H-8), 6.70 (1H, d, J = 2.0 Hz, H-6), 5.34 (1H, d, J = 7.8 Hz, H-1"), 3.25-3.75 (6H, m, H-2" to H-6").

Peonidin 3-glucoside (P3G): Q-TOF/MS: *m/z* 463.1247 $[M]^+$ (calcd 463.1240 for $C_{22}H_{23}O_{11}^+$), 301.0715 $[M\text{-}Glc]^+$; ^{1}H NMR [500 MHz, MeOH-d_4-TFA-d_1 (9 : 1, v/v)] δ 9.06 (1H, *s*, H-4), 8.25 (1H, *dd*, J = 8.6, 2.2 Hz, H-6'), 8.23 (1H, *d*, J = 2.2 Hz, H-2'), 7.06 (1H, *d*, J = 8.6, Hz, H-5'), 6.92 (1H, *d*, J = 1.8 Hz, H-8), 6.67 (1H, *d*, J = 2.0 Hz, H-6), 5.31 (1H, *d*, J = 7.7 Hz, H-1''), 4.01 (3H, s, -OMe) 3.93-3.44 (6H, m, H-2" to H-6").

Results and Discussion

As shown in Fig. 2A, HPLC analysis showed that

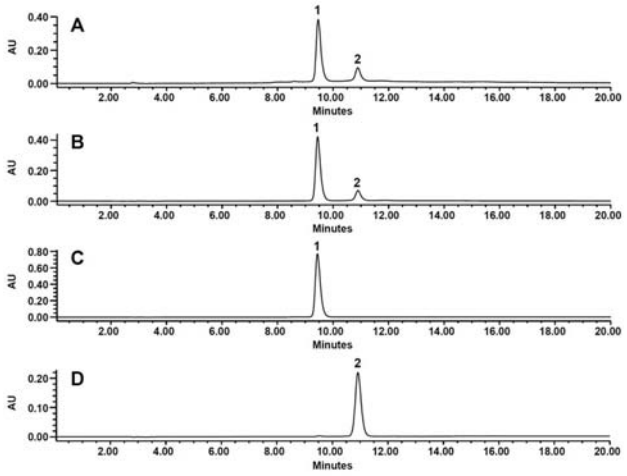

Fig. 2. HPLC chromatogram of crude black rice extract (**A**); enriched anthocyanin extract prepared by HPCCC (**B**); C3G (**C**) and P3G (**D**) isolated by RP-MPLC. Peak **1**: C3G; Peak **2**: P3G. [HPLC condition: column - Eclipse XDB-C18 (4.6 × 250 mm I.D., 5 μm); mobile phase - see Experimental; detection wavelength - 520 nm; injection volume - 20 μl].

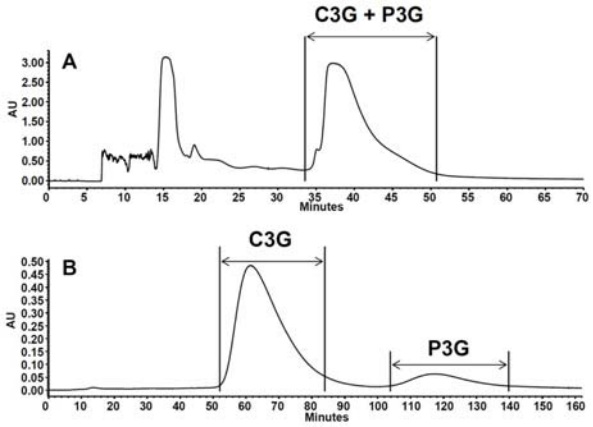

Fig. 3. HPCCC/UV chromatogram of crude black rice extract (**A**); RP-MPLC/UV chromatogram of enriched anthocyanin extract (**B**). [HPCCC condition: two-phase solvent system - TBME/B/A/0.01% TFA (1 : 3 : 1 : 5, v/v); rotational speed - 1600 rpm; mobile phase flow rate - 4.5 ml/min; sample amount - 500 mg; detection wavelength - 520 nm. RP-MPLC condition: mobile phase - MeOH-Water (30 : 70, v/v, acidified with 0.1% TFA); flow rate - 20 ml/min; sample amount - 37.4 mg; detection wavelength - 520 nm].

crude BRE contained C3G and P3G as major component which is good accordance with previously published literatures. The amount of C3G and P3G in BRE (500 mg) was determined to be 21.7 and 14.9 mg, respectively, by in-house quantitative HPLC analysis.

Usually, preparing enriched anthocyanin extract for further isolation have been performed using multi-step solid-support based column chromatography such as Sephadex LH-20, Amberlite-XAD and ion exchange resins,[9-11] but the efficiency of these methods was not satisfactory because target anthocyanins were co-eluted with polar impurities e.g. sugars and organic acids and low recovery rate. In addition, anthocyanins are susceptible to degradation by light, oxygen, pH and temperature,[12] thus time consuming complicated column chromatography should be avoided in anthocyanin separation process.

In this study, HPCCC was employed to prepare enriched anthocyanin extract directly from crude BRE. A two-phase solvent system composed of TBME/B/A/0.01% TFA (1 : 3 : 1 : 5, v/v, reversed-phase mode) was used because TBME/B/A/W/TFA systems were applied to CCC method for the isolation of diverse anthocyanins.[13] Five hundred mg of BRE was subjected to HPCCC to yield enriched anthocyanin extract (37.4 mg, Fig. 2B and Fig. 3A) and it was eluted in 35 - 50 min. The amounts of C3G and P3G in enriched anthocyanin extract were evaluated to be 20.4 and 13.1 mg, respectively, showing the recovery rate of 94.0% (C3G) and 87.9% (P3G).

Finally, enriched anthocyanin extract (37.4 mg) was chromatographed on RP-MPLC (Fig. 3B) to isolate pure C3G (16.5 mg, recovery rate: 76.0%, Fig. 2C) and P3G (8.7 mg, recovery rate: 58.3%, Fig. 2D) with the purities of 98.2% and 96.3%, respectively. The structures of isolated C3G and P3G were confirmed by Q-TOF/MS and ^{1}H NMR spectroscopic data as well as comparing them with previously published reports. In MS spectrum, C3G showed the molecular ion peak at 449.1094 $[M]^+$ corresponding to $C_{21}H_{21}O_{11}^+$ (calcd for 449.1078) and fragment ion peak at 287.2558 $[M\text{-}Glc]^+$, while mass peak of P3G were shown at 463.1247 $[M]^+$ and 301. 0715 $[M\text{-}Glc]^+$. The ^{1}H NMR values of C3G and P3G were in good agreement with published values.[14,15] Consequently, the overall separation was achieved in a day with high purities and recovery rates of C3G and P3G, which indicated that HPCCC coupled with RP-MPLC method is more rapid and efficient than multi-step conventional column chromatography for the separation of anthocyanins.

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