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Original Article

Composition of anthocyanins in mulberry and their antioxidant activity

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ABSTRACT

Anthocyanins in the fruits of mulberry (*Morus alba* L.) were extracted and separated by high-speed counter-current chromatography (HSCCC) using a biphasic solvent system composed of methyl tert-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:3:1:5:0.001) to yield five anthocyanins: cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-D-glucopyranoside) (C3RG), cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-D-galactopyranoside) (C3RGa), cyanidin 3-*O*-β-D-glucopyranoside (C3G), cyanidin 3-*O*-β-D-galactopyranoside (C3Ga) and cyanidin 7-*O*-β-D-glucopyranoside (C7G), respectively. The five compounds were identified by ESI–MS and one/two-dimensional NMR spectra. The antioxidant activity of crude mulberry anthocyanins (CMA), C3G, C3Ga, C7G, C3RG and C3RGa was investigated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method. The results showed that CMA, C3G, C3Ga, C3G and C7G have higher scavenging ability on DPPH. At the concentration of 0.10 mg/mL, the DPPH radical scavenging rates of C3G, C3Ga and C7G were about 88% of vitamin C, while C3RG and C3RGa were about 60% of it. CMA had the same DPPH radical scavenging rate as vitamin C or the five anthocyanin monomers when the concentration reached 0.40 mg/mL, which shows that CMA is an excellent antioxidant agent.

1. Introduction

Anthocyanins are the water-soluble pigments in plants, which contribute to the brilliant color of blue, red and purple of leaves, flowers and fruits. Anthocyanins represent a class of important antioxidants, as they are so common in human foods. In recent years, many papers have been published on the in vitro antioxidant activity of anthocyanins and their other functions, as well as studies assessing the correlation between their antioxidant capacity and chemical structure (Kong et al., 2003). Also, a lot of studies have shown that anthocyanins may have potential effects in reducing the risk of cardiovascular diseases and cancers by antioxidant, anti-inflammatory and chemoprotective properties (Tsuda et al., 1994, 1998; Meiers et al., 2001; Bagchi et al., 2004; Lazze et al., 2004). In addition, the anthocyanins possess potent inhibition effects on the epidermal growth-factor receptor (Tsuda et al., 1999) and neuroprotective effects on the PC12 cells exposed to hydrogen peroxide in vitro and on cerebral ischemic damage in vivo (Kang et al., 2006). Mulberries are anthocyanins-rich fruits, which are a traditional Chinese medicine, used for dizziness and blurred vision. Mulberry anthocyanins also exhibit inhibitory effect on the migration and invasion of a human lung cancer cell line (Chen et al., 2006). Though the published papers reported that cyanidin 3-*O*-β-D-glucopyranoside (C3G) and cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-D-glucopyranoside (C3RG) are the main anthocyanins in mulberry (Liu et al., 2004; Dugo et al., 2001; Lee et al., 2004), the anthocyanin compounds in mulberry are not fully identified by NMR spectral data yet. In the present study, the anthocyanins in mulberry were extracted, separated and identified, and the antioxidant activities of five anthocyanin monomers and crude mulberry extracts (CME) were assayed.

2. Experimental

2.1. Materials

All solvents for extraction and separation were of analytical grade, purchased from Hangzhou Huadong Chemicals Inc., China. The mulberries, belonging to the fruits of *Morus alba* L, were purchased at a local fruit store in Hangzhou, China. The mulberries (10 kg) with similar color (dark red) were picked for extraction of anthocyanins. The fresh fruits were directly used for extraction without cleaning and homogenizing. Ascorbic acid (Vitamin C) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (Shanghai Division).

2.2. Extraction of mulberry anthocyanins

The fresh mulberries (10 kg) were extracted with 50 L of methanol–formic acid (9:1) at room temperature for 24 h twice. The combined extract was evaporated to a syrup. The syrup was dissolved in 1000 mL of 0.01 mol/L HCl, and was defatted with

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1200 mL of ethyl acetate. The water solution was subjected to column chromatography of Amberlite XAD-7 orderly eluted with water and methanol–formic acid (9:1) (Degenhardt et al., 2000). The methanol–formic acid elution solution was evaporated into a syrup and freeze-dried to yield 65 g of crude mulberry anthocyanins (CMA).

2.3. Separation of mulberry anthocyanins

The separation of mulberry anthocyanins was performed by high-speed counter-current chromatography (HSCCC) (Du et al., 2004). The high-speed countercurrent chromatograph used in the present study was constructed at the Institute of Food and Biological Engineering, Zhejiang Gongshang University (Hangzhou, China). The apparatus was equipped with a 1200 mL column with six-layer coils made of 5.0 mm i.d. teflon tubing. The separation system was composed of a K-1800 Wellchrom pump (Knauer, Germany), a 100 mL sample loop made of 3 mm i.d. polytetrafluoroethylene (PTFE) tubing, the high-speed countercurrent chromatograph and a B-684 collector (Büchi, Switzerland). For the HSCCC separation of CMA, the solvent system was composed of methyl tert-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:3:1:5:0.001, v/v), and the organic upper phase was used as the stationary phase. The sample solution was prepared by dissolving 1.0 g of CMA in 100 mL of the mobile phase. For each separation, the coil column was first entirely filled with the stationary phase. Then, the apparatus was rotated at 700 rpm and the sample solution was injected into the HSCCC system through the PTFE sample loop with the mobile phase at a flow rate of 3.0 mL/min. The mode for HSCCC separation was "head to tail". The effluent was monitored at 520 nm by Elite UV-200 detector (Elite, Dalian, China) and collected into 15-mL tubes.

2.4. Analytical controls and structure elucidation

2.4.1. HPLC analysis of anthocyanins

HPLC separation was performed on an YMC-Pack ODS-AQ, S-3 μm, 150 × 4.6 mm^2 column (YMC Separation technology, Kyoto, Japan). Gradient elution was carried out with the solvent system A (water–formic acid–acetonitrile, 87:10:3, v/v) and system B (water–formic acid–acetonitrile, 40:10:50, v/v) at a flow rate of 0.8 mL/min. The gradient steps were set from 6% B to 20% B in 20 min, to 40% B in 15 min, to 60% B in 5 min, to 70% B in 5 min, at 70% B for 6 min and back to initial conditions (Du et al., 2004).

2.4.2. Electrospray ionization mass spectrometry (ESI-MS)

All ESI-MS experiments were performed on a Bruker Esquire LC–MS ion trap multiple mass spectrometer (Bremen, Germany) in positive ionization mode analyzing ions up to *m*/*z* 2200. During ESI-MS studies, the pure HSCCC fractions II, III, IV, V and VI were directly introduced via a syringe pump at a flow rate of 0.24 mL/min. The drying gas was nitrogen (gas flow 7.0 L/min, 330 °C), and the nebulizer pressure was set to 34.5 kPa. ESI-MS parameters (positive mode): capillary, −4500 V; end plate, −4000 V; cap exit, +90 V; cap exit offset, +60 V; skim 1, +30 V; skim 2, +10 V.

2.4.3. Nuclear magnetic resonance (NMR) analysis

^1H, ^{13}C and DEPT 90/135 NMR spectra, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlations (HMBC), total correlation spectroscopy (TOCSY), rotating frame overhause effect spectroscopy (ROESY) and H–H correlation spectroscopy (^1H–^1H COSY) were recorded in [$^2H_4$] methanol (MeOH-d_4)-[$^2H_1$] trifluoroacetic acid (TFA-d_1) (19:1) on a Bruker Avance 500 (Karlsruhe, Germany) with 500 MHz for ^1H measurements and 125 MHz for ^{13}C measurements, respectively.

2.5. Evaluation of antioxidant activity

The radical scavenging activity was performed by the DPPH assay (Brand-Willams et al., 1995; Rotondi et al., 2004) to evaluate the antioxidant activity of the purified components from mulberry. The sample solutions of 0.8 mg/mL were prepared by dissolving 4.0 mg of each sample in 5 mL of water. Those sample solutions were diluted into sample solutions of 0.4, 0.2 and 0.1 mg/mL. The sample solutions were stored in a refrigerator at 4 °C before testing for radical scavenging activity. In the test protocol of radical scavenging activity, 2.9 mL of 65 μM DPPH reagent in methanol/water (80:20, v/v) was added to 0.1 mL of sample solution to be tested. After 30 min of reaction at 25 °C, the absorbance was measured at 515 nm and compared to a control sample prepared with 0.1 mL of methanol/water (80:20, v/v) solution without adding samples. The radical scavenging activity was expressed as percentage of DPPH radical elimination calculated according to the following equation:

$$R_s(\%) = (A_0 - A_1)/A_0 \times 100\%$$

where R_s is the radical scavenging activity of a sample, A_0 is the absorbance of the sample at reaction time $t = 0$ min and A_1 is the absorbance of the sample at $t = 30$ min of the reaction. All tests were run in triplicate, and the average value was calculated. The analysis of variance was performed if the effect of treatment was significant at minimal 5% of probability ($P < 0.05$).

3. Results and discussion

3.1. Anthocyanins in mulberry

Preparative isolation of monomeric anthocyanins by HSCCC requires solvent systems of high polarity. The two-phase solvent system composed of tert-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:3:1:5:0.001, v/v) was selected for HSCCC separation. In routine separation the aqueous phase is used as the mobile phase, and for stabilization of anthocyanin pigments addition of 0.1% trifluoroacetic acid is necessary. Fig. 1 presents the chromatogram of the HSCCC separation of 1.0 g CMA. The HSCCC run yielded fractions of six separated peaks which were combined to fraction I (231–255 min), fraction II (296–355 min), fraction III (376–400 min), fraction IV (456–525 min), fraction V (606–710 min) and fraction VI (866–1065 min), respectively. Evaporation of the organic solvents under reduced pressure, and subsequent lyophilization yielded 270 mg of component I, 57 mg of component II, 7 mg of component III, 233 mg of component IV, 33 mg of component V and 19 mg of component VI. HPLC analysis (Fig. 2) indicated that component I was a mixture which gave three peaks at 280 nm and one peak at 520 nm, while components II–VI yielded single peaks at 280 and 520 nm, which indicates they were possibly monomeric anthocyanins. The purity of components II–VI was more than 95%, calculated based on the peak area at 280 nm. Components II–VI, corresponding to compounds **1**–**5**, were subjected to ESI–MS and one/two dimensional NMR analysis to identify their chemical structure (Fig. 3).

Compound **1** yielded ions *m*/*z* 595 and 449 (positive) corresponding to the molecular ion and cyanidin-glucose in its ESI-MS spectrum. The downfield region of the ^1H NMR spectrum of **1** showed a 3H ABX system at δ 9.16 (s, H-4), δ 7.12 (s br, H-8) and δ 6.91 (d, 2.0 Hz; H-6) and a 3H AMX system at δ 8.49 (dd, 9.0, 2.5 Hz; H-6′), δ 8.24 (d, 2.0 Hz; H-2′) and δ 7.24 (d, 8.5 Hz; H-5′) (Table 1), which suggested cyanidin aglycone in **1**. The sugar regions of the ^1H and ^{13}C NMR spectra of **1** were in accordance with one rhamnose and one glucose unit (Byamukama et al., 2005). All the ^1H sugar