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Effect of air-drying temperature on antioxidant capacity and stability of polyphenolic compounds in mulberry (*Morus alba* L.) leaves

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ABSTRACT

The mulberry (*Morus alba* L.) leaf is a promising dietary source of antioxidants such as quercetin due to its relatively high content of that compound. We investigated effects of an air-drying process on the antioxidant capacity and stability of antioxidant polyphenolic compounds in mulberry leaves. Main compounds playing a central role in antioxidant activities in mulberry leaves are quercetin glycosides and chlorogenic acid. Raw mulberry leaves were air-dried at various temperatures, and antioxidant activity using DPPH radical scavenging assay and levels of antioxidant compounds were measured. DPPH radical scavenging activity and levels of polyphenolic compounds in mulberry leaves air-dried at 60 °C or below were not significantly different from those of freeze-dried mulberry leaves, whereas both values in mulberry leaves air-dried at 70 °C and over decreased significantly. These results indicate that strict temperature control is important in the production of mulberry leaf products to maintain antioxidant activity and levels of polyphenolic compounds.

1. Introduction

Mulberry (*Morus alba* L.) leaves, bark and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure (Jia, Tang, & Wu, 1995). In Japan, consumption of mulberry leaves as a tea or powdered juice has been increasing. Several recent studies reported the antioxidant activity of mulberry leaves. Butanol extract of mulberry leaves scavenged the DPPH radical and inhibited the oxidative modification of rabbit and human LDL (Doi, Kojima, Makino, Kimura, & Fujimoto, 2001). Five flavonol glycosides – rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempferol 3-(6-acetylglucoside), and chlorogenic acid have been reported in mulberry leaves as antioxidants (Matsuoka, Kimura, & Muraoka, 1994; Onogi et al., 1993). We identified quercetin 3-(6-malonylglucoside) as the quantitatively major antioxidant compound in mulberry leaves (Katsube et al., 2006). We also showed that mulberry leaves attenuated development of atherosclerotic lesions in LDL receptor knockout mice through enhancement of LDL resistance to oxidative modification, and these antioxidative and antiatherogenic protective effects were mainly attributed to quercetin 3-(6-malonylglucoside) (Enkhmaa et al., 2005). As the mulberry leaf is a promising dietary source of antioxidants such as quercetin due to its relatively high content of that compound, it is important to study the processing conditions of mulberry leaves in the production of mulberry leaf products to best preserve the antioxidant activity and effective levels of polyphenolic compounds. The drying of mulberry leaves is necessary and commonly used in making tea and most other mulberry leaf products. Two methods mainly used for drying of perishable foods are air-drying and freeze-drying. Generally, air-drying is favored due to processing cost and speed. Drying processes may affect the quality and quantity of antioxidant activity in mulberry leaves, but this has never been clarified. The purpose of the present study was to study effects of a drying process on the antioxidant capacity and levels of antioxidant polyphenolic compounds in mulberry leaves.

2. Materials and methods

2.1. Materials

Mulberry (*M. alba* L.) leaves were harvested in Sakurae-cho, Gotsu City, Shimane Prefecture, Japan, in June 2007. Dried mulberry leaves produced by Sakurae Kuwatya Seisan Kumiai (Shimane, Japan) were used for purification of antioxidant compounds. Quercetin 3-(6-malonylglucoside) was purified from the leaves as described previously (Katsube et al., 2006). Rutin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), chlorogenic acid, Trolox

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and ethanol were obtained from Wako Chemicals, Ltd. (Osaka, Japan), and isoquercitrin and astragalin from Funakoshi Co., Ltd. (Tokyo, Japan). Acetonitrile for chromatographic analysis was of analytical grade and purchased from Sigma–Aldrich Japan K.K. (Tokyo, Japan).

2.2. Extraction procedure of antioxidant compounds in mulberry leaves

Generally, ethanol or methanol solutions containing some water, particularly those ranging from 40% to 80% ethanol or methanol, are more efficient in the extraction of polyphenolic compounds than pure water, ethanol or methanol (Suzuki et al., 2002). In our previous study, a 60% ethanol solution proved to be the most efficient in extracting flavonol glycosides from the mulberry leaves (Katsube et al., 2006). Therefore, we used 60% ethanol solution for extraction of the antioxidant compounds in the present study. To extract the antioxidant compounds, 100 mg of mulberry leaves in a dry powder form were suspended in 10 mL of 60% (v/v) ethanol aqueous solution and stirred with a magnetic stir-bar for 2 h at 40 °C in a water bath. After centrifugation at 13,000*g* for 10 min, the extracted solution was filtered through a 0.45 μm filter (ADVANTEC MFS, Inc., Tokyo, Japan) and used for measurement of antioxidant activity and levels of polyphenolic compounds.

2.3. DPPH radical scavenging activity

Antioxidant activity of the crude extract was evaluated by DPPH radical scavenging assay (Gouki et al., 2006). Briefly, 50 μL of the 60% ethanol leaf extract prepared as described before, 50 μL of 40% ethanol aqueous solution (v/v), and 50 μL of 0.2 M-morpholinoethanesulfonic acid (MES) buffer at pH 6.0 were placed in a 96-well microplate. The leaf extract was diluted with 60% ethanol aqueous solution. The reaction was initiated by adding 50 μL of 800 μM DPPH in ethanol. After left standing for 20 min at room temperature, the reaction mixture's absorbance at 540 nm was measured with a Multilabel Counter (PerkinElmer, Inc., Wellesley, USA). DPPH radical scavenging activity was expressed as mmol Trolox equivalent/100 g of dry matter using the standard Trolox curve. This extraction of the dried mulberry leaves was repeated three times, and antioxidant activity was measured for each extraction solution and represented as average ± SD. Antioxidant activity of individual polyphenolic compounds in the mulberry leaves was also evaluated by DPPH radical scavenging assay as described above and expressed as relative Trolox equivalent per mol.

2.4. HPLC analysis for quantitative determination of antioxidant compounds in mulberry leaves

Several antioxidant compounds in mulberry leaves have been previously reported (Katsube et al., 2006; Matsuoka et al., 1994; Onogi et al., 1993). Chlorogenic acid, rutin, isoquercitrin, quercetin 3-(6-malonylglucoside) and astragalin in mulberry leaves were analyzed by a quantitative HPLC system (LaChrom, Hitachi, Ltd. Tokyo, Japan), by comparisons with standard compounds using ODS-80Ts column (4.6 × 250 mm) (Tosoh Corporation, Tokyo, Japan); solvent, acetonitrile/0.1% formic acid (20:80); UV detection, 280 nm (0–7.5 min) for detection of chlorogenic acid and 370 nm (7.5–30 min) for detection of flavonols, velocity of a fluid, 1 mL/min. A typical HPLC profile of 60% ethanol aqueous extract of mulberry leaves is shown in Fig. 1. An unidentified compound which showed a fairly low peak (Fig. 1, peak number 7) was noted, and we purified this compound and analyzed its structure, as we describe in Section 2.5.

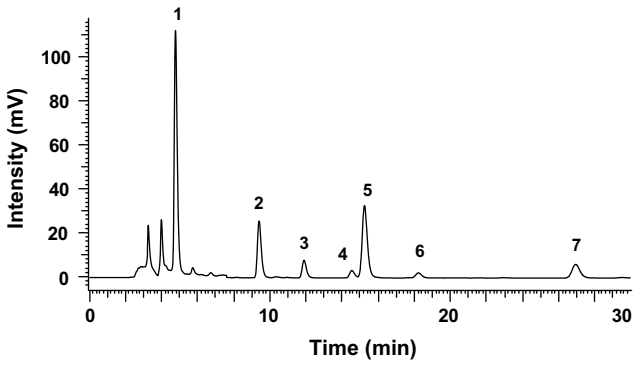

Fig. 1. Typical HPLC chromatogram of phenolic compound extract from mulberry leaves. Peaks: 1, chlorogenic acid; 2, rutin; 3, isoquercitrin; 4, unknown; 5, quercetin 3-(6-malonylglucoside); 6, astragalin; 7, kaempferol 3-(6-malonylglucoside).

2.5. Isolation of antioxidant compound ("compound A") from mulberry leaves

One hundred grams of dried mulberry leaves were extracted two times with 1 L of 60% (v/v) ethanol solution. The resulting extract was concentrated to dryness under reduced pressure, and the residue was dissolved in water (100 mL) and partitioned with ethyl acetate (3 × 100 mL). These aqueous fractions were combined and concentrated to dryness under reduced pressure, and the residue was dissolved in water and filtrated using a 0.45 μm filter. This aqueous fraction was loaded into a preparative chromatography system (AKTA purifier, GE Healthcare UK Ltd. Buckinghamshire, UK) using an ODD-80Ts column (21.5 × 300 mm) (Tosoh Corporation); solvent, acetonitrile/0.1% formic acid (20:80); flow rate, 10 mL/min; UV detection, 370 nm. Peak fractions corresponding to the target compound (Fig. 1, peak number 7) were pooled and further purified by recrystallization which produced 160 mg of a yellow powder we labeled as "compound A".

2.6. Hydrolysis reactions of compound A

Acid hydrolysis of compound A was done. About 100 μg of compound A were mixed with 2 N hydrochloric acid in a total volume of 2 mL and incubated at 60 °C for 2 h. The reaction mixture was concentrated to dryness under reduced pressure, and the residue was dissolved in 50% (v/v) ethanol solution. The resulting flavonol aglycon was analyzed using LC-APCI-MS (LCQ DECA XP, Thermo Fisher Scientific, Inc., Waltham, USA), and the sugar component was analyzed using a Carbo Pac PA1 column of a DX-500 liquid chromatograph (Dionex, Co., Sunnyvale, USA) as previously described (Katsube, Yamasaki, Iwamoto, & Oka, 2003).

2.7. APCI-MS and NMR of compound A

APCI mass spectra of compound A were obtained using a LCQ DECA XP (Thermo Fisher Scientific, Inc.): negative mode: *m*/*z* 533 $[M\text{-}H]^-$.

^{13}C NMR (70 MHz, DMSO-d6): δ 177.3 (C-4), 167.7 (malonate CO_2H), 166.5 (CO malonate CO_2R), 164.2 (C-7), 161.2 (C-5), 160.0 (C-4), 156.6 (C-9), 156.4 (C-2), 133.1 (C-3), 130.8 (C-2', C-6'), 120.7 (C-1'), 115.0 (C-3', C-5'), 103.9 (C-10), 101.1 (C-1 glucose), 98.7 (C-6), 93.7 (C-8), 76.1 (C-3 glucose), 74.0 (C-2 glucose), 73.8 (C-5 glucose), 69.5 (C-4 glucose), 63.5 (C-6 glucose), 41.0 (malonate CH_2).

2.8. Drying treatment

The raw mulberry leaves were washed with tap water and spin-dried to remove surface water. The leaves were divided into