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Analytical Methods

Flavonoid analysis of buckwheat sprouts

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ABSTRACT

It is known that common buckwheat sprouts contain several flavonoids, including orientin, isoorientin, vitexin, isovitexin, rutin, and quercetrin, whereas tartary buckwheat sprouts contain only rutin. In this study, we evaluated flavonoids present in buckwheat sprouts and identified a previously unreported flavonoid. Simultaneous detection by HPLC was used to separate rutin and a compound that was not separated in previous studies. We used a novel HPLC elution gradient method to successfully separate rutin and the previously unidentified compound, for which we performed structural analysis. The identification of six flavonoids by HPLC was confirmed using HPLC–ESI–MS/MS analysis. The newly identified compound, $[M + H]^+ = 611.17$, was identified by NMR as the rutin epimer quercetin-3-*O*-robinobioside. Unlike common buckwheat sprout, tartary buckwheat sprout contained rutin as a main flavonoid, whereas other flavonoids appeared only in trace amounts or were not detected. Quercetin-3-*O*-robinobioside was not detected in tartary buckwheat sprout.

1. Introduction

Buckwheat (*Fagopyrum* spp.), a member of the Polygonaceae family, is consumed all around the world. While it is technically a fruit seed, it is classified as a cereal grain because its utilisation is similar to that of other cereal grains (Kim, Kim, & Park, 2004). Common buckwheat (*Fagopyrum esculentum* Möench) and tartary buckwheat (*Fagopyrum tataricum* Gaertner) are the main species of buckwheat consumed by humans. Buckwheat has been established as a nutritional food because of its abundant levels of amino acids and protein (Bonafaccia, Marocchini, & Kreft, 2003). Moreover, common buckwheat is a major dietary source of rutin and *O*-glycosyl flavonols including both quercetin and rutinose moieties (Kitabayashi, Ujihara, Hirose, & Minami, 1995).

The nutritional values of the edible parts of sprouts of bean sprouts, cereal crops, and vegetables have gained interest in recent years. Sprouts are recognised as outstanding dietary vegetables in Asia, Europe, and the United States, and are an important source of protein, mineral, dietary fiber, and vitamins in human diets. Especially, the contents of polyphenols, which are secondary plant metabolites, in particular, increase or are newly synthesised during sprouting, similar to peanut and broccoli (Guo, Yuan, & Wang, 2011; Wang et al., 2005). Polyphenols have attracted a great deal of attention due to their various benefits for human health. For example, the total phenolic content and antioxidant capacity of mungbean sprouts is greater than that of seeds (Kim, Jeong, Gorinstein, & Chon, 2012). Likewise, the flavone and flavonol glycoside contents of buckwheat increase during sprouting (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010). Buckwheat sprouts are one of the most popular forms of sprouts and are consumed as both a salad vegetable and fresh vegetable served with noodles in northeast Asia (Kim et al., 2004).

Phytochemical analyses of buckwheat sprouts have been performed intensively over the last decade, and the nutritional components of seeds have been evaluated for significantly longer. Compared with buckwheat seeds, the sprouts contain relatively large amounts of rutin, which is known as a beneficial compound for health (Kim et al., 2004). Similarly, buckwheat sprouts have a significantly greater abundance of other flavonoids including orientin, isoorientin, vitexin, isovitexin, rutin, and quercetrin compared with buckwheat seeds (Kim et al., 2004, 2008; Lim, Park, Kim, Jeong, & Kim, 2012; Liu, Chen, Yang, & Chiang, 2008; Watanabe, 2007). However, the analytical high performance liquid chromatography (HPLC) techniques employed in previous studies were unable to separate and quantify individual flavonoids to a satisfactory degree. In addition, the HPLC chromatograms of numerous studies are ambiguous as to whether they represent single or multiple peaks.

Reversed-phase HPLC is widely employed for quantitative and qualitative analysis of common buckwheat sprouts, and numerous

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studies have used this approach for identification of flavonoids. Several studies have reported that common buckwheat sprouts contain *C*-glycosyl flavones (orientin, isoorientin, vitexin and isovitexin) and rutin (Kim et al., 2008; Lim et al., 2012; Liu et al., 2008; Watanabe, 2007). In this study, we used a gradient elution, based on water-acetonitrile, to achieve optimum separation for the identification of flavonoid derivatives in common buckwheat sprouts.

In order to identify a newly detected compound, we used NMR, HPLC, and Q-TOF MS to isolate and identify the quercetin derivative, *O*-glycosyl flavonol, in addition to the other flavonoids and their derivatives.

2. Materials and methods

2.1. Seeds

Common (Cultivar, Suwon 2 Ho) and tartary (Cultivar, Dae-Guan 3-3 Ho) buckwheat seeds were purchased from a seed company (Budnara) in Gwangju, Republic of Korea in 2012.

2.2. Chemicals

Orientin ($\geqslant$95%), isoorientin ($\geqslant$95%), vitexin ($\geqslant$95%), isovitexin ($\geqslant$98%), and rutin ($\geqslant$94%) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All solvents used were of analytical or HPLC grade.

2.3. Sprout growth condition

Buckwheat seeds were placed in a 4 °C room before initiation of experiments. After soaking in distilled water for 4 h, water saturated-seeds were placed in a dark growth chamber for 48 h to facilitate germination. Germinated seeds were planted in a light supplemented growth chamber (35 μmol m^{-2} s^{-1} light intensity by fluorescent light lamps) at 25 °C for 5 days. During sprout growth, water was supplied by spraying as needed. Finally, the sprouts were harvested and dried in a 30 °C dry oven equipped with ventilation for 3 days.

2.4. Extraction and fractionation of flavonoids from buckwheat sprouts

Fifteen grams of dried buckwheat sprouts were processed with a commercial grinder and dispersed in 150 mL of 70% aqueous ethanol. After extraction (48 h) at room temperature, the solution was filtered through Whatman #2 filter paper (Whatman International Limited, Kent, UK). The solid filter cake was extracted again in 70% aqueous ethanol and filtered. The extraction procedure was repeated once more. Next, solvent was evaporated using a rotary evaporator at 40 °C, and 840 mg of crude extract was suspended in 40 mL of distilled water and combined with 80 mL of EtOAc/*n*-BuOH (3:1). After shaking to mix solvents, the mixture was allowed to separate and split again between water and EtOAc/*n*-BuOH. A total of 250 mg of EtOAc/*n*-BuOH fractionated extract was gathered after solvent evaporation. The extract (250 mg) was suspended in 20 mL of water and combined with 40 mL of EtOAc. After repeating the separation described above, the aqueous fraction was collected and dried to obtain a 70 mg flavonoids-rich extract, which was kept at 4 °C until analysed.

2.5. Isolation of flavonoids in buckwheat sprouts by reversed-phase HPLC

Flavonoids of buckwheat sprouts were analysed using a reversed-phase HPLC system (Shimadzu model, Kyoto, Japan) equipped with an auto-sampler (SIL-20A, Shimadzu), photodiode array detector (SPD-20A, Shimadzu), binary pump (LC-20AD, Shimadzu), and vacuum degasser. Chromatographic separation was performed using a ProntoSIL 120-5-C18-ace-EPS column (4.6 × 250 mm, 5.0 μm; Bischoff, Leonberg, Germany). For HPLC analysis, we used two different linear solvent gradients with a binary mobile phase consisting 0.1% HCOOH in water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B). Elution gradient I consisted of 92% A and 8% B at 0 min, 85% A and 15% B at 4 min, 55% A and 45% B at 90 min, 5% A and 95% B at 100 min, 92% A and 8% B at 102 min, and 92% A and 8% at 105 min. Elution gradient II consisted of 92% A and 8% B at 0 min, 85% A and 15% B at 4 min, 84% A and 16% B at 8 min, 84% A and 16% B at 20 min, 83% A and 17% B at 45 min, 82% A and 18% at 75 min, 81% A and 19% at 80 min, 80% A and 20% B at 95 min, 79% A and 21% B at 102 min, 30% A and 70% B at 107 min, 92% A and 8% B at 110 min and 92% A and 8% B at 115 min. The flow rate was at 0.8 mL/min with a 20 μL injection volume. The column oven temperature was set to 25 °C, and the absorbance was monitored at 350 nm (Li et al., 2007). The extracts were analysed at least three times by HPLC.

2.6. HPLC-DAD–ESI/Q-TOF MS analysis of flavonoids in common buckwheat sprouts

The molecular weights of major flavonoids in buckwheat sprouts were determined using an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight MS (Q-TOF LC/MS) with an Agilent 1200 Series Rapid Resolution LC system. The MS was operated with electrospray ionisation source in either negative or positive ion mode. A ProntoSIL 120-5-C18-ace-EPS column (4.6 × 250 mm, 5.0 μm; Bischoff, Leonberg, Germany) was used to separate individual flavonoids using gradient elution II at an oven temperature of 40 °C. Flavonoids were monitored at 350 nm. The MS source parameters were as follows: 350 °C drying gas (N_2) temperature, 12 L/min drying gas flow, 45 psi nebulizer pressure, 200 V fragmentation voltage, and 4000 V capillary voltage. MS and MS/MS ranges were set from *m/z* 50 to 1700 and *m/z* 50 to 1100, respectively. Collision energy was set at 10 eV for positive ion mode and 40 eV for negative ion mode.

2.7. Prep-LC and TLC analysis for flavonoid separation

Flavonoids from the crude extract of common buckwheat sprouts were separated and confirmed using a preparative high-performance liquid chromatography system (*Prep-LC*) consisting of a Waters 2555 Prep-LC controller, a 2998 Photodiode array detector, and an Empower workstation (Waters, Milford, MA, USA) with a YMC ODS column (20 × 250 mm, 5 μm). The binary solvent system consisted of (A) water and (B) acetonitrile, with a linear gradient of 5% (B) to 50% (A) for 10 min, isocratic for 10 min, from 50% (B) to 100% (A) for 10 min, isocratic for 10 min, and finally from 100% (B) to 5% (A) for 5 min. The flow rate was 30 mL/min and 70 mg/mL of flavonoid-rich extract was injected with 1 mL injection volume. The column oven temperature was set to 25 °C. In addition, we performed thin-layer chromatography (TLC) analysis to confirm the purity of isolated fractions. TLC analysis was carried out using Kiesel gel 60 F_{254} and RP-18 F_{254S} resin (Merck, Dramstadt, Germany). TLC spots were detected using a Spectroline Model ENF-240 C/F UV lamp (Spectronics Corporation, Westbury, NY, USA) and a 10% H_2SO_4 solution.

2.8. NMR spectroscopy

The structure of a previously unidentified flavonoid was analysed by NMR. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR