The Contribution of Polyphenols to Antioxidative Activity in Common Buckwheat and Tartary Buckwheat Grain

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Abstract: We examined the contribution of polyphenols to the antioxidative activity in the grains of common buckwheat “Hitachi akisoba” (H) and “Kanto No.1” (K) and in those of Tartary buckwheat “Rotundatum” (R) and “Pontivy” (P). The antioxidative activity in the 80% ethanol extracts was 16.4 and 15.3 μmol-Trolox g⁻¹ DW in H and K, respectively, and 52.9 and 57.4 μmol-Trolox g⁻¹ DW in R and P, respectively. These extracts were analyzed by HPLC. In common buckwheat, (-)-epicatechin, (-)-epicatechingallate, and rutin were confirmed. The (-)-epicatechin content was 20.2 and 15.6 mg 100 g⁻¹ DW, and those of rutin were 13.6 and 12.2 mg 100 g⁻¹ DW in H and K, respectively. (-)-Epicatechin accounted for about 13 and 11% of the total antioxidative activity in H and K, respectively, and rutin about 2% in both varieties. Since each polyphenol accounted for only about one fifth of the total antioxidative activity, the existence of unknown antioxidants was suggested. In Tartary buckwheat, rutin, quercitrin, and quercetin were confirmed. The rutin content was 1808.7 and 1853.8 mg 100 g⁻¹ DW, in R and P, respectively. Rutin accounted for about 90 and 85% of the total antioxidative activity in R and P, respectively. Accordingly, rutin appears to be the major antioxidant in Tartary buckwheat.

Key words: Antioxidative activity, Common buckwheat, Contribution, Polyphenol, Tartary buckwheat.

The genus Fagopyrum includes ten-odd species (Hirose and Ujihara, 1998). Among them, common buckwheat (Fagopyrum esculentum Moench.) and Tartary buckwheat (Fagopyrum tartaricum Gaertn.) have been cultivated for their value as food ingredients. Buckwheat is well known as a healthy food because it contains large amounts of protein and minerals. Recently, with the progress of food science, the functional effect of buckwheat has been clarified.

Rutin is especially interesting to researchers because it prevents the elevation of blood pressure (Matsubara et al., 1985). Following this discovery, numerous genetic resources were analyzed, and common buckwheat grains was found to contain about 10-30 mg 100 g⁻¹ DW rutin, while Tartary buckwheat grains about 1-2 g 100 g⁻¹ DW rutin (Suzuki et al., 1987; Kitabayashi et al., 1995a, b; Ohsawa and Tsutsumi, 1995; Morishita and Tetsuka, 2002). Furthermore, high-rutin common buckwheat varieties were also developed (Minami et al., 2001; Ito et al., 2005).

On the other hand, buckwheat grain has a higher antioxidative activity than other cereal grains (Zieliński and Kozlowska, 2000). A highly antioxidative variety is also in demand because antioxidants protect the human body from oxidative damage caused by free radicals and help fend off lifestyle and adult diseases. Rutin has been considered to be a potentially major antioxidant because it shows antioxidative activity (Suzuki and Miyazawa, 1991). Oomah and Mazza (1996) reported that there was a significant correlation between antioxidative activity and flavonoid content; however, this is not the case for rutin in common buckwheat. There is a significant correlation, however, antioxidative activity and total polyphenol content (Watanabe et al., 1995; Morishita et al., 2002). Various antioxidative compounds, such as vitamins B1, B2, and E, and phenolic compounds (polyphenols: rutin, quercetin, proanthocyanidines, etc.) have been identified in common buckwheat hulls and groats (Watanabe et al., 1995, 1997; Watanabe, 1998). In particular, Watanabe (1998) reported that catechins contributed to the antioxidative activity in common buckwheat groats. However, the degree of the contribution of each polyphenol to antioxidative activity has not been reported. Moreover, there have been no reports about the relationship between antioxidative activity and polyphenols in Tartary buckwheat. It is necessary to determine the contribution of each polyphenol to antioxidative activity for efficient breeding of a highly antioxidative variety.

In this study, the contribution of each polyphenol to antioxidative activity was estimated for common buckwheat and Tartary buckwheat.

Materials and Methods

1. Plant materials and preparation of buckwheat flour

Two common buckwheat varieties “Hitachi akisoba” (H) and “Kanto No.1” (K) and two Tartary buckwheat varieties “Rotundatum” (R) and “Pontivy” (P) were
sown in an experimental field at the Institute of Radiation Breeding on August 21, 2003. The block size was 2.4 × 2 m, and the plant density was 25.6 plant · m² (6.5 × 60 cm). Each variety had three replications in a randomized block design and was harvested at maturity. After harvest, the seeds were dehulled and milled with a mortar and pestle. The obtained flour was stored at −30°C until extraction.

2. Preparation of buckwheat flour extracts and measurement of water content
Buckwheat flour (0.6 g) was extracted with 6 ml of an 80% ethanol solution warmed to 80°C for 30 min in a screw-capped test tube. After cooling, the test tube was centrifuged at 3,000 rpm for 10 min. The obtained supernatant was used for HPLC analysis. One-fifth of the supernatant of common buckwheat diluted using 80% ethanol and 1/25 of the diluted supernatant of Tartary buckwheat were used for the measurement of antioxidative activity. Residual flour was dried at 105°C for 3 hours to measure the water content.

3. Measurement of antioxidative activity of buckwheat grain
The antioxidative activity was estimated from the DPPH radical-scavenging activity (Morishita et al., 2002). Reagent 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Wako Pure Chemical Industries, Ltd., Japan) was dissolved in 99.5% ethanol at a concentration of 0.4 mM (W/V). The reaction mixture consisted of 0.3 ml of 0.4 mM DPPH solution, 0.3 ml of a 200 mM 2-morpholinoethanesulfolic acid (MES) (Funakoshi, Ltd., Japan) buffer (pH 6.0), 0.3 ml of 20% ethanol, 0.24 ml of 80% ethanol, and 0.06 ml of a diluted buckwheat flour extract. The reaction was started by the addition of the diluted extract. After the test tube had been allowed to stand for 20 min, the absorbance of the reaction mixture was measured at 520 nm (Hitachi U-3000, Hitachi, Inc., Japan). A reaction was conducted simultaneously using 80% ethanol (blank) or Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich Com., USA) (standard) instead of the buckwheat flour extract. A Trolox solution was prepared by dissolving in 80% ethanol at concentration of 0.2 mM. The DPPH radical-scavenging activity was determined from the decrease of absorbance at 520 nm and expressed as a μmol-Trolox g⁻¹ DW using a calibration line of Trolox.

4. Polyphenol analysis by HPLC
Buckwheat extracts were analyzed by high-performance liquid chromatography (HPLC) according to the procedure reported by Horie et al. (2001) after modification. The HPLC systems and conditions are shown in Table 1. A 10 μl aliquot of samples was used for the quantitative analysis of polyphenols. Some of the detected peaks were confirmed by comparing the retention times and spectrum patterns of the external standards of standard reagents using a photo-diode array detector. (-)-Epicatechin and (-)-epicatechingallate were

| Apparatus       | Shimadzu LC-10A                          |
|-----------------|-----------------------------------------|
| Detector        | SPD-M10A/Dp (photo-diode array detector) |
| Wavelength      | 280 nm, 360 nm                          |
| Column          | SHISEIDO CAPCELLPAK C18                 |
| Mobile phase    | A: 2.5% Acetic acid, Methanol, Acetonitrile (400 : 0 : 10) |
|                 | B: 2.5% Acetic acid, Methanol, Acetonitrile (400 : 400 : 10) |
| Gradient program| 0−2 min A : B = 95 : 5                  |
|                 | 2−48 min A : B = 95 : 5 → 5 : 95       |
|                 | 48−54 min A : B = 5 : 95               |
|                 | 54−56 min A : A = 5 : 95 → 95 : 5      |
|                 | 56−60 min A : B = 95 : 5               |
| Flow rate       | 1.0 ml min⁻¹                           |
| Column temperature | 40°C                        |

Table 1. HPLC system and conditions.

| [Common buckwheat] | [Tartary buckwheat] |
|--------------------|---------------------|
| Hitachi akisoba    | 16.4 ± 0.6          |
| Kanto No.1          | 15.3 ± 0.7          |
| Rotundatum          | 52.9 ± 0.8          |
| Pontivy             | 57.4 ± 1.6          |

Unit : μmol-Trolox g⁻¹ DW. 3 rep. AVG ± SD.

Table 2. Antioxidative activities in common buckwheat and Tartary buckwheat varieties.
Fig. 1. HPLC patterns of 280 nm and 360 nm in common buckwheat “Hitachi akioba” and Tartary buckwheat “Rotundatum”.
detected at 280 nm. Rutin, quercitrin, and quercetin were detected at 360 nm. The concentrations of each compound were calculated by comparing the peak areas of samples with those of standard reagents.

### 5. Estimation of the contribution of antioxidative activity in each polyphenol contained in flour

Each of the standard reagents, rutin and quercetin (Wako Pure Chemical Industries, Ltd., Japan) and (-)-epicatechin, (-)-epicatechingallate, and quercitrin (quercetin-3L-rhamnoside) (Funakoshi, Ltd., Japan), was dissolved in 80% ethanol and analyzed for its specific antioxidative activity by using the method of DPPH radical-scavenging activity with the aforementioned procedure. The rate of contribution of the antioxidative activity of each polyphenol to the total antioxidative activity was calculated by multiplying the specific antioxidative activity of each polyphenol and the total polyphenol content of buckwheat grain.

### Results

Table 2 shows the antioxidative activity of the grains of common buckwheat and Tartary buckwheat varieties. In common buckwheat, H and K showed activity of 16.4 and 15.3 μmol-Trolox g⁻¹ DW, respectively. In Tartary buckwheat, R and P showed activity of 52.9 and 57.4 μmol-Trolox g⁻¹ DW, respectively. The antioxidative activity of Tartary buckwheat was 3-4 times higher than that of common buckwheat.

Fig. 1 shows the HPLC patterns at 280 nm and 360 nm in common buckwheat H and Tartary buckwheat R. At 280 nm, numerous peaks were detected in both common and Tartary buckwheat. In the case of common buckwheat, (-)-epicatechin, (-)-epicatechingallate and rutin were confirmed by comparing the retention times and spectrum patterns of the external standards of standard reagents. On the other hand, in Tartary buckwheat, rutin, quercitrin, and quercetin were confirmed. Among them, rutin had the largest peak. Neither (-)-epicatechin nor (-)-epicatechingallate were detected. Most of the detected peaks were unknown in both common and Tartary buckwheat.

Table 3 shows the content of each polyphenol in the grains of common and Tartary buckwheat. In the common buckwheat varieties H and K, the (-)-epicatechin content was 20.2 and 15.6 mg 100 g⁻¹ DW, (-)-epicatechingallate content was 2.4 and 1.3 mg 100 g⁻¹ DW (P < 0.01), and rutin content was 13.6 and 12.2 mg 100 g⁻¹ DW, respectively. H had higher contents of these polyphenols than K, and (-)-epicatechingallate content was significantly different between H and K by t-test. The rutin content in Tartary buckwheat, R and P was 1808.7 and 1853.8 mg 100 g⁻¹ DW (near 2 g 100 g⁻¹ DW), quercitrin content was 95.4 and 81.2 mg 100 g⁻¹ DW (P < 0.05), and quercetin content was 2.0 and 2.4 mg 100 g⁻¹ DW, respectively. The difference in quercitrin content between R and P was significant by t-test.

Fig. 2 shows the total antioxidative activity and the contribution of each polyphenol contained in the grain in the total antioxidative activity. In common buckwheat, (-)-epicatechin was the major contributor of antioxidative activity, and its value was about 2 μmol-Trolox g⁻¹ DW. In H and K, (-)-epicatechingallate accounted for about 13 and 11%, of the total antioxidative activity, respectively. The contribution rate of rutin was about 2%. That of (-)-epicatechingallate was almost the same as that of rutin. On the other hand, in Tartary buckwheat, the contribution of rutin was approximately 50 μmol-Trolox g⁻¹ DW, and it accounted for about 90 and 85% of the total antioxidative activity, respectively. Rutin accounted for most of the antioxidative activity in Tartary buckwheat grain.

### Discussion

In this study, the contribution of polyphenols to antioxidative activity in common and Tartary buckwheat grain was investigated. Several peaks were detected by HPLC in both of common and...
Tartary buckwheat, and among them (-)-epicatechin, (-)-epicatechingallate, rutin, quercitrin, and quercetin were confirmed from the retention time and spectrum pattern compared with standard reagents (Fig. 1). These compounds were contained in buckwheat grains and showed antioxidative activity (Watanabe et al., 1997; Watanabe, 1998; Fabjan, 2003). Consequently, we measured these polyphenols in this study.

Tartary buckwheat grains had 3–4 times higher antioxidative activity than common buckwheat grains (Table 2), and the rutin content of Tartary buckwheat was more than 100 times that of common buckwheat, in agreement with previous reports (Kitabayashi et al., 1995a, b; Ohsawa and Tsutsumi, 1995; Morishita and Tetsuka, 2002). Suzuki and Miyazawa (1991) reported that rutin shows antioxidative activity. Consequently, it was surmised that the contribution of polyphenols to antioxidative activity is different between common and Tartary buckwheat. In fact, not only rutin but also other polyphenol components were different between common and Tartary buckwheat (Table 3). Quercetin was not detected in common buckwheat because of the low activity of the rutin-degrading enzyme, which catalyzes rutin to quercetin and rutinose (Yasuda et al., 1992; Morishita et al., 1998). Furthermore, this reaction was inhibited by the low water content of flour (10–12%), storage at −30°C from milling to extraction, and by ethanol and high temperature (80°C) during extraction. Rutin has been considered as an important antioxidant since the report by Suzuki and Miyazawa (1991). However, Oomah and Mazza (1996) reported that there was no significant correlation between antioxidative activity and rutin content in common buckwheat. Our study revealed that the contribution rate of rutin to antioxidative activity was about 2% of the total activity, suggesting that rutin is not a major antioxidant in common buckwheat grain (Fig. 2). Some investigators found a significant correlation between antioxidative activity and polyphenol content (Watanabe et al., 1995; Morishita et al., 2002). Watanabe (1998) reported that (-)-epicatechin, was a major antioxidant and its content was higher than that of rutin in common buckwheat groats. Our study also revealed that (-)-epicatechin accounted for a large part of the antioxidative activity in common buckwheat grain (the contribution rates of H and K were about 13 and 11%, respectively) (Fig. 2). However, the sum of the contribution rate of all confirmed antioxidants ((-)-epicatechin + (-)-epicatechingallate + rutin) was only about one-fifth of the total antioxidative activity. Therefore, there are probably other unidentified antioxidants contributing to the antioxidative activity in common buckwheat grain. For example, the contribution of the antioxidant vitamin E (tocopherol), as estimated from the reported data on contents (Honda, 1995) and specific antioxidative
activity (Suda et al., 2003), is lower than that of rutin. However, quantitative data about other antioxidants is not available. On the basis of the HPLC pattern, the residual antioxidative activity (about four-fifths of the total antioxidative activity) may result from the contribution of various unidentified antioxidants.

In Tartary buckwheat, the contribution of rutin to antioxidative activity in R and P was about 90 and 85%, respectively. From the HPLC patterns, many unidentified peaks were detected as well as in common buckwheat, but the ratio of the rutin area to the total area at 280 nm and 360 nm was also about 90 and 85%, respectively (data not shown). Consequently, rutin appears to be the most important antioxidant in Tartary buckwheat.

In the future, it will be necessary to define the roles of the unidentified compounds. Furthermore, the correlations among each antioxidant ((-)-epicatechin, (-)-epicatechingallate, and rutin) and antioxidative activity should be determined. Our findings are expected to be useful for breeding buckwheat varieties with higher antioxidative activity.

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