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Anti-oxidative analysis, and identification and quantification of anthocyanin pigments in different coloured rice

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ABSTRACT

Anthocyanin pigments in coloured rice cultivars were isolated and identified using high-performance liquid chromatography techniques. Two black rice cultivars (Asamurasaki, Okunomurasaki) contained three major anthocyanins: cyanidin-3-glucoside, peonidin-3-glucoside and malvidin. Chinakuromai (black) rice additionally contained a fourth anthocyanin, petunidin-3-glucoside. Four red rice cultivars contained only malvidin. The total anthocyanin content varied greatly among black rice cultivars (79.5–473.7 mg/100 g), but was lower in red rice (7.9–34.4 mg/100 g). Total phenolic content was similar between red (460.32–725.69 mg/100 g) and black (417.11–687.24 mg/100 g) rice. The oxygen radical absorbing capacity was ranked as follows: red (69.91–130.32 μmol Trolox/g) > black (55.49–64.85 μmol Trolox/g) > green (35.32 μmol Trolox/g) > white (21.81 μmol Trolox/g) rice. The antioxidant capacity resulted mainly from the seed capsule, not the endosperm. The anthocyanin pigments contributed little to the total antioxidant capacity of red (0.03–0.1%) and black (0.5–2.5%) rice cultivars. Hence, the antioxidant capacity is derived mainly from other phenolic compounds.

1. Introduction

Rice (*Oryza sativa* L.) is consumed as a staple food by over half the world's population, and the main cultivation regions include South Asia, China, Korea, Thailand, and Japan. Rice is generally categorised by colour into red, green, black, and white (common) varieties, and this is determined by the composition of anthocyanin pigments that are ubiquitous throughout the plant kingdom. Anthocyanin pigments are flavonoids, a group of phenolic compounds that play an important biological role in reducing the risk of oxidative damage, cancer, and cardiovascular disease (Acquaviva et al., 2003; Harborne, 1997; Harborne & Williams, 2000; Koide, Kamei, Hasimoto, Kijoma, & Haseqawa, 1996; Lazze et al., 2003; Osawa, Ramarathnam, Kawakishi, & Namiki, 1992, chap. 10; Russo et al., 2005).

A considerable number of studies have been conducted in the last three decades to examine the antioxidant activity of black rice (Havsteen, 1983; Hiemori, Koh, & Mitchell, 2009; Hu, Zawistowski, Ling, & Kitts, 2003; Laokuldilok, Shoemaker, Jongkaewwattana, & Tulyathan, 2011; Ling, Cheng, Ma, & Wang, 2001; Toyokuni et al., 2002). However, few such reports exist concerning red rice, and none has determined the antioxidant characteristics of green rice. Further, there is a paucity of data comparing antioxidant activity between different coloured rice strains. Though coloured rice is rich in phenolic acids, including anthocyanins, there is little published information on whether phenolic content affects antioxidant capacity (Zhang, Zhang, Zhang, & Liu, 2010).

There are several reports that focus on identification of anthocyanin pigments in black rice cultivars, but few have explored red rice varieties. A number of studies have shown that the main anthocyanin pigments of black rice are cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) (Abdel-Aal, Young, & Rabalski, 2006; Hu et al., 2003; Tian, Giusti, Stoner, & Schwartz, 2005). Yao, Sang, Zhou, and Ren (2010) reported that black rice contained petunidin-3-glucoside (Pt3G). In red rice, the situation is not so clear: Abdel-Aal et al. (2006) reported that C3G was the main anthocyanin, whereas Kim et al. (2008) concluded that red rice did not contain anthocyanin pigments.

In this study, we used an oxygen radical absorbing capacity (ORAC) assay to compare the antioxidant activities in the caryopsis and endosperm of different strains of coloured rice, as well as between cultivars of the same strain. We also examined total phenolic content (with UV spectrophotometry) and anthocyanin content (with high performance liquid chromatography) in the cultivars of four rice strains, and identified the major anthocyanin pigments present.

2. Materials and methods

2.1. Materials

Three strains of coloured rice and one common cultivar (white rice) were selected for the following study. One white rice cultivar

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(Nakateshinsenbon), four red rice cultivars (Benisarasa, Tsukushiakamochi, Beniroman and Tohboshi), three black rice cultivars (Okunomurasaki, Chinakuromai and Asamurasaki) and one green rice cultivar (Akunemochi) were used. The rice was cultivated in the experimental fields of the Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, and harvested in 2009. Seeds were stored at 4 °C until use.

2.2. Chemicals

Authentic anthocyanin standards (C3G, P3G, Pt3G, malvidin and cyanidin) were purchased from Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). AAPH (2,2-azobis-2-amidino-propane dihydrochloride), Folin–Ciocalteu's reagent, gallic acid monohydrate and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonsaure) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Hiroshima, Japan), and were of the highest purity available.

2.3. Sample preparation

Randomly selected caryopsis from each coloured cultivar was milled using an ultra centrifugal mill (Iwatani, Osaka, Japan). Milled rice powder (2.0 g) was soaked in 1% HCl methanol or 80% methanol, and sequentially extracted for 6 h at room temperature with shaking. The total volume of extract was adjusted to 50 ml with 1% HCl methanol or 80% methanol. Samples extracted with 1% HCl methanol were used for the ORAC assay or the HPLC. Samples extracted with 80% methanol were used for measurement of phenolic content. Endosperm samples of the coloured rice cultivars, polished to 90% by a rice polisher (Panasonic, Tokyo, Japan), were analysed using the ORAC assay, and the extraction method employed was the same as for caryopsis. All extracted samples were stored at −80 °C until use.

2.4. Oxygen radical absorbing capacity (ORAC) assay

The assays determining antioxidant capacity were carried out on a Thermo Labsystems Fluoroskan Ascent FL plate reader (Sigma Chemical Co.). The temperature of the incubator was set at 37 °C. The procedure employed was based on the method of Wu et al. (2004) and Huang, Ou, Maureen, Flanagan, and Prior (2002). In brief, AAPH was used as the peroxyl radical generator, Trolox as the standard, and fluorescein as the fluorescent probe. With the use of appropriate filters, an excitation wavelength of 485 nm was selected, and fluorescence emission at 520 nm was measured every 2 min over a 90-min period. The measurements were taken in triplicate. The final ORAC results were standardized to Trolox. The fluorescence analysis system automatically generated Trolox standard curves using net fluorescence (background-subtracted values). The relative Trolox equivalent ORAC value was calculated as follows: relative ORAC value = $(AUC_{sample} - AUC_{blank})$ micromoles of Trolox equivalents per gram of dry weight/$(AUC_{Trolox} - AUC_{blank})$ micromoles of Trolox equivalents per gram of dry weight. The data were analysed using a Microsoft Excel macro program (Microsoft, Roselle, IL, USA).

2.5. Measurement of total phenolic content (TPC)

TPC was determined using a slight modification of the Folin–Ciocalteu method (Zhang et al., 2010). In brief, to generate a standard curve, 0.05 g gallic acid monohydrate was dissolved in 80% methanol and adjusted to 50 ml with 80% methanol. Aliquots (0.025, 0.05, 0.10, 0.20, 0.40 and 0.60 ml) of the 50 ml gallic acid monohydrate solution were transferred to cuvettes, and 1.25 ml of Folin–Ciocalteu reagent and 3.75 ml of 20% Na_2CO_3 solution were added to each cuvette. The solution was then incubated at 30 °C for 2 h. A solution without gallic acid monohydrate was used as the control. The TPC was calculated by the absorbance at 760 nm using an UV spectrophotometer (DU 530, Beckman Coulter, Brea, CA, USA).

2.6. Measurement of total anthocyanin content (TAC) and identification of anthocyanins by high-performance liquid chromatography (HPLC)

Separation of anthocyanins was carried out with an HPLC system equipped with an InertSustain C18 (5 μm, 250 × 4.6 mm ID) column and a VIS 535 nm (LC800 UV) detector (Hitachi, Tokyo, Japan), using an injection volume of 20 μl, and operated at room temperature with a flow rate of 1 ml/min. Elution was carried out using a gradient system with $H_2O/CH_3CN/CH_3OH/HCOOH$ = 40/22.5/22.5/10, v/v/v/v (solvent A) and $H_2O/CHOOH$ = 90/10, v/v (solvent B) as follows: 7 min, 7% A and 93% B; 35 min, 25% A and 75% B; 10 min, 65% A and 35% B. Five authentic anthocyanin standards, C3G, P3G, Pt3G, malvidin and cyanidin (Tokiwa Phytochemical Co., Ltd.), were used to allow quantitative analysis of anthocyanin pigments in the various rice samples. The peak times of the various rice samples were compared to those of the standard anthocyanins (to allow identification), and HPLC was used to quantify the results for each detected anthocyanin in each rice sample. Individual experiments were repeated three times to ensure reproducibility.

2.7. Statistical analysis

All measurements from the same extract were carried out in triplicate in order to determine reproducibility. Analysis of variance was used to determine differences in antioxidant activity, TAC or TPC. Statistical significance was accepted at $P < 0.05$, determined using a *t*-test.

3. Results and discussion

3.1. Antioxidant capacity of the caryopsis

The antioxidant capacity of one white, one green, four red, and three black rice cultivars was evaluated using the ORAC assay (Table 1). The results showed that the antioxidant capacity of rice followed the rank order: red rice cultivars (69.91–130.32 μmol Trolox/g) > black rice cultivars (55.49–64.85 μmol Trolox/g) > green rice (35.32 μmol Trolox/g) > white rice (21.81 μmol Trolox/g). Furthermore, significant differences were observed between the antioxidant capacities of individual cultivars within red coloured rice. For example, the red rice cultivar Tsukushiakamochi showed twofold greater antioxidant capacity than Tohboshi. In contrast, the value did not markedly vary among the three black rice cultivars. In a comparison of red, black, and purple rice cultivars, Yao et al. (2010) reported that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of black rice was greater than that of red rice; similar results were reported by Laokuldilok, Shoemaker, Jongkaewwattana, and Tulyathan (2011). In contrast, Oki et al. (2002) reported that the DPPH radical scavenging activity of red-hulled rice was greater than that of black and white-hulled rice. However, our data showed that the antioxidant capacities of two of the four red rice cultivars were significantly greater than those of the black rice cultivars, as determined by the ORAC assay. It is possible that the genetic makeup of rice, as well as environmental factors during cultivation, led to the observed differences in antioxidant capacity.