Journal of Cereal Science 51 (2010) 28–34

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

A study of biodiversity of flavonoid content in the rice caryopsis evidencing simultaneous accumulation of anthocyanins and proanthocyanidins in a black-grained genotype

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ARTICLE INFO

Article history:
Received 29 June 2009
Received in revised form
4 September 2009
Accepted 7 September 2009

Keywords:
Pigmented rice
Total antioxidant capacity
Proanthocyanidins
Anthocyanins

ABSTRACT

Rice genotypes with pigmented caryopses have received increased attention because of their antioxidant properties. Previous works evidenced that the kernel of red rice is characterized by the presence of proanthocyanidins, whereas black rice is characterized by the presence of anthocyanins. In the present study, the total antioxidant capacity (TAC) and the polyphenol content of the caryopsis were evaluated on a set of Italian rice varieties (three white, two black and five red ones). The pigmented rices, on average, had a TAC four times higher than the white ones. As expected, red-grained genotypes contained no detectable anthocyanins and one black rice contained no detectable proanthocyanidins. However, the black-grained cv. Artemide had large amounts of both proanthocyanidins and anthocyanins. This genotype was also characterized by the highest TAC and polyphenol content: its TAC was about twice the TAC of the other pigmented rices, and it had a polyphenol content 2–3 times the content found in the other pigmented rices. Pigmented genotypes are confirmed to be very interesting to breed rice for high polyphenol content and TAC. Furthermore, the possibility to select for genotypes accumulating both anthocyanins and proanthocyanidins provides a way to substantially increase the polyphenol content and TAC of the rice caryopsis.

1. Introduction

Rice genotypes with either red or purple/black bran layer have been cultivated for a long time in Asia (Ahuja et al., 2007). It may be worthy to mention that, even if the term 'red rice' is commonly used to designate a weedy rice, cultivated rices with a red caryopsis (actually, red-brick colour) also do exist (Ahuja et al., 2007). Since rice is harvested as a covered caryopsis (paddy rice), the kernel colour is directly visible only after removing the hull. Pigments are accumulated in a single layer of cells in the seed coat of the immature caryopsis just above the nucellar cuticle (Krishnan and Dayanandan, 2003). This inner seed coat persists below the pericarp in the caryopsis of pigmented rice when the seed matures and the other integument layers are crushed and reabsorbed (Krishnan and Dayanandan, 2003).

Although rice is one of the most important cereal crops in the world, pigmented rice varieties are cultivated only in restricted areas of the globe, where they are appreciated because of a long lasting tradition (Ahuja et al., 2007). Notwithstanding their low diffusion, in the recent years pigmented rice varieties have received increased attention because of their antioxidant properties (Abdel-Aal et al., 2006; Ahuja et al., 2007; Chung and Shin, 2007; Finocchiaro et al., 2007; Hu et al., 2003; Jang and Xu, 2009; Nam et al., 2006; Oki et al., 2002; Shen et al., 2009; Zhang et al., 2006). Experimental studies have reported that coloured rice supplementation decreases oxidative stress *in vivo* and simultaneously increases antioxidant capacity *in vivo* and *in vitro* (Lin and Weng, 2006; Ling et al., 2001; Toyokuni et al., 2002; Xia et al., 2003). Such healthy properties have been related to several classes of antioxidant compounds present in rice, including tocols, oryzanols and phenolic compounds (Finocchiaro et al., 2007; Ling et al., 2001; Sugano and Tsuji, 1997; Xu et al., 2001).

Phenolics are antioxidants, and there is a general belief that the phenolics present in plant food contribute to prevent the oxidative damage that is implicated in a range of diseases, including cancer, cardiovascular diseases and aging (Lin and Weng, 2006; Scalbert and Williamson, 2000; Scalbert et al., 2005). However, polyphenols

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ANOVA, analysis of variance; TAC, total antioxidant capacity; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; PA, proanthocyanidins.

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doi:10.1016/j.jcs.2009.09.003

are poorly absorbed and are extensively metabolized in the intestine, liver and possibly brain tissue (Aura, 2008; Ghosh and Sheepens, 2009). Thus, the modest levels of polyphenols in the blood or brain are unlikely to be high enough to neutralize free radicals chemically, but rather, they may stimulate intracellular signaling pathways leading to vascular protection aside from their direct chemical antioxidative properties (Ghosh and Sheepens, 2009; Virgili and Marino, 2008). Accordingly, Schewe et al. (2008) hypothesized that dietary flavonoids may act as antioxidants *in vivo* in a more broad sense by interfering with prooxidant processes or by inhibition of prooxidant enzymes. As an alternative mechanism for their health effects, it has been suggested that flavonoids regularly introduced with the diet can act as mild prooxidants and stimulate the endogenous antioxidant defences, which prevents disease development or reduces the impact of oxidative stress when disease occurs (Moskaug et al., 2005).

Different actions should however be expected for different classes of phenolics (depending on bioavailability after food treatments, metabolization and excretion), and their effects *in vivo* need further investigations (Aron and Kennedy, 2008; Cermak et al., 2009; Duthie et al., 2003; Fardet et al., 2008; Geleijnse and Hollman, 2008; MacGhie and Walton, 2007; Virgili and Marino, 2008).

Previous work evidenced that the kernel of red rice is characterized by the presence of proanthocyanidins (PA) (Finocchiaro et al., 2007; Oki et al., 2002; Reddy et al., 1995), whereas black rice is characterized by the accumulation of anthocyanins, mainly cyanidin-3-glucoside and peonidin 3-glucoside (Abdel-Aal et al., 2006; Hu et al., 2003; Jang and Xu, 2009; Reddy et al., 1995; Ryu et al., 1998; Zhang et al., 2006). Both classes of flavonoids are plant-defense chemicals which act against pathogens by interfering with enzyme activity (Reddy et al., 1995; Shirley, 1998); moreover, they are powerful antioxidants (Aron and Kennedy, 2008; MacGhie and Walton, 2007). However, they differ in several properties: anthocyanins are monomeric and mostly glycosylated, whereas proanthocyanidins are oligomeric/polymeric (Aron and Kennedy, 2008; MacGhie and Walton, 2007). In addition, the former are coloured *per se*, at least at acidic pH (Castañeda-Ovando et al., 2009), whereas the latter have to be oxidized into complex compounds to give the red colour (Finocchiaro et al., 2007; Lepiniec et al., 2006).

In the present study the total antioxidant capacity and the polyphenol content of a set of Italian rice varieties (three white, two black and five red ones) was evaluated to establish the existing genetic variability for these parameters. Spectrophotometric assays were used to distinguish the main classes of compounds that characterise the kernel of the pigmented rice.

2. Experimental

2.1. Rice materials

Five red-grained rice genotypes (RNC1, RNC3, RNC5, Perla Rosso and Ermes), two black-grained rice genotypes (Venere, Artemide) and three Italian white rice varieties (Augusto, Perla, Gladio) were harvested in 2005 and 2006 in an experimental field in Vercelli (Northern Italy). The different grains are shown in Fig. 1, and their characteristics are reported in Table 1. Length to width ratio and grain type were determined on the dehulled caryopses according to the U.S.D.A. Rice Inspection Handbook (1997).

All the samples were milled with a Cyclotec Sample Mill (Foss Italia S.p.A., Padova, Italy) equipped with a 0.5 mm screen. The moisture content was determined with a Precisa HA60 IR Moisture Analyzer (Precisa Instruments, Diekinton, Germany). Moisture content of dehulled rice samples ranged from 10.0% to 15.0% (mean value of 12.0%). Samples were stored at −20 °C before analysis. All the analyses were carried out in duplicate for 1–2 field replications.

2.2. Total antioxidant capacity assay (TAC)

To assay the antioxidant capacity of antioxidant compounds that are not covalently linked to cellular components, and do not require, for the extraction, a preliminary hydrolysis, rice samples (0.5 g) were extracted with 5 mL of methanol for 20 min on a horizontal shaker (220 oscillations/min) at room temperature. Supernatant was separated by centrifugation at 1000×*g* for 10 min and the extraction was repeated twice. The three supernatants were combined. The residue was re-extracted by the addition of 5 mL of acetone/water (70:30, v/v), centrifuged at 1000×*g* for 10 min and the supernatant was recovered. This extraction was repeated twice and supernatants were combined. The residue was further used to extract the bound phenolic compounds (i.e. polyphenols that are covalently linked to cellular components, Perez-Jimenez and Saura-Calixto, 2005). For this, the residue was digested with 10 mL of 2 M NaOH at room temperature for 1 h. The extract was then adjusted to pH 3 with 3 M acetic acid. The antioxidants released during the basic hydrolysis were extracted with 10 mL of ethyl acetate. After centrifugation at 1000×*g* for 10 min, the supernatant was collected and the extraction repeated twice. Then, the solvent was evaporated under vacuum and the residue recovered by adding deionized water. All rice extracts were adequately diluted (depending on their activity) in the corresponding solvent and immediately analyzed for their antioxidant capacity.

The TAC was evaluated according to the Trolox equivalent antioxidant capacity (TEAC) method based on ABTS radical cation decoloration in the presence of antioxidants (Pellegrini et al., 1999). The ABTS radical cation was prepared by reacting a 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration), allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The solution was then diluted in absolute ethanol to an absorbance of 0.70 ± 0.20 AU (Absorbance Units) at 734 nm. Results were expressed in mmol of Trolox (used as the antioxidant reference) per kg of dry matter. The total antioxidant capacity (TAC) was determined as the sum of the antioxidant capacity of methanol, acetone/water and hydrolyzed extracts. The variation in values of antioxidant capacity between analytical replicates was always between 3 and 10% relative standard deviation (RSD). When the RSD was higher than 10%, the analyses were repeated to confirm the value.

2.3. Determination of total polyphenol content

To measure total polyphenols, milled samples of dehulled rice (200 mg) were extracted for 2 h with 2 mL of 2 M NaOH, vortexing every 10 min. Total polyphenols were quantified with the modified Prussian blue assay (Graham, 1992). Briefly, 0.1 mL of the rice extracts and 3 mL of distilled water were dispensed into test tubes. Then, 1 mL of 0.016 M $K_3Fe(CN)_6$ and 1 mL of 0.02 M $FeCl_3$ were added in rapid succession. After 15 min, 5 mL of stabilizer (30 mL distilled water, 10 mL 85% H_3PO_4, 10 mL 1% arabic gum) were added to each sample. Absorbance was measured at 700 nm by using a DU-64 spectrophotometer (Beckman Instruments, CA, USA). Increasing concentrations of catechin standard (between 0.3 and 1.0 mmol/L, initial concentration) were used to build up a calibration curve. As we verified the linearity of the curve in the range found in our samples, the assay was standardized against 0.001 M catechin and polyphenols were expressed as g per kg (of dry matter) of catechin equivalents.

The Prussian blue colorimetric assay is a redox-based test: phenolic compounds reduce iron in ferricyanide to produce