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Total anthocyanin content and antioxidant activity of germinated colored rice

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Abstract: The objective of this study was to determine effect of germination on total anthocyanin content (TAC) and antioxidant activity of colored rice (*Oryza sativa* L). The normal, non-waxy rice variety Phitsanulok 2 was used as a control and two pigmented rice including black glutinous rice (local name Niew Dam), and black non-waxy rice (local name Hom Nil) were used in this study. Rough rice and dehulled rice kernels were steeped in water for 12 and 6 hrs, respectively before further left to germinate for 0, 6, 12, 18, and 24 hr. Rice samples were evaluated for TAC, trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP). Rice grains without husk showed higher germination rate compared to those of rough rice. After germination, the results showed that TAC and TEAC of black glutinous rice were significantly higher than that of the other two samples. Black rice germinated from rough rice showed higher TAC content and TEAC than that germinated from grains without husk. Therefore, rice with husk intact should be employed for the preparation of germinated pigmented rice to protect TAC loss during germination process.

Keywords: Germination, antioxidant, pigmented rice, anthocyanin, TEAC

Introduction

The most common rice consumed by humans is white rice, followed by brown rice; however rice genotypes with either red, purple or black bran layer have been cultivated for a long time in Asia (Ahuja *et al*., 2007). Colored rice posses unique color and flavor, therefore they are used as ingredient in many dishes (Rhee *et al*., 2000). However, due to the limitation in term of hard texture of cooked colored rice, they are not popular for consumption even though it has been long known about the beneficial effects of pigment in these groups of rice. In pigmented rice, there are naturally occuring color substances that belong to the flavonoid group called anthocyanins. Positive health effects of the pigments present in the bran layer of rice have been reported. A commonly found anthocyanin in colored rice is acelylated procyanidins, which is reported to possess a free radical scavenging activity (Oki *et al*., 2002)

Recently, a germination process is introduced to cereal and legume seeds and many studies have reported its advantages and health benefits. In a germination process, rice has been softened during the steeping stage, which will be soft enough to eat when cooked in an ordinary rice cooker. Germinated rice contain numerous nutrients include γ-aminobutyric acid (GABA), dietary fiber, inositols, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc, γ-oryzanol, and prolylendopeptidase inhibitor (Shoichi, 2004). Beneficial biological activities of these compounds have been well documented. Regular intake of germinated brown rice has been found good for health; e.g., it can help to prevent headache, colon cancer, heart disease, and Alzheimer's disease, as well as lower blood pressure and regulate blood sugar level (Kayahara and Tukahara, 2000)

Usually, germinated rice is made from normal rice (*Oryza sativa* L.) containing brownish bran with the popular cultivar such as fragrance rice (local name Khao Dawk Mali105), and Phatumthani variety. Unfortunately, scientific data relevant to germinated rice, especially black rice, are not readily available in Thailand. In addition, there is no existing study to validate the combined effect of germination and the pigment of rice in terms of antioxidative property change. This research, therefore, introduced an alternative choice of using black rice as a raw material for making germinated rice. Two pigmented rice including black glutinous rice (local name Niew Dam), and black non-waxy rice (local name Hom Nil), which are popular rice cultivars for consumption in the lower north area of Thailand, were selected for the present study.

As the consumers become more health conscious and more aware of the benefits of functional foods, diets containing bioactive compounds such as antioxidants have received greater attention. Therefore, to address the gap of knowledge on germinated rice, especially black rice, this study was conducted to determine the effect of germination on the antioxidant content and its activity of germinated black rice.

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Materials and Methods

Chemicals

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and trolox were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents used were of analytical grade.

Rice samples

The rice samples comprised of 3 varieties of rice (*Oryza sativa* L.) including a non-pigmented rice (Phitsanulok 2 variety) and 2 pigmented rice (black waxy rice or Niew Dam and black non-waxy rice or Hom Nil variety). All rice samples were harvested from Pichit province, Thailand.

Germination of rice samples

Two forms of rice were prepared; (1) Rough rice (grains with husk intact) and (2) dehulled rice (grains with husk removed). Germination process was conducted according to previously described by Jiamyangyuen and Ooraikul (2008). Briefly, rice grains (100 g) were steeped in water at room temperature for 12 hrs and 6 hrs for rough rice and dehulled rice, respectively. Water was drained off and grains were washed again with water. Grains were then left to germinate in the dark at room temperature for 0, 6, 12, 18, and 24 hrs. After germination, the rice grains were dried at 50°C to maintain moisture content <13%. The dried grains were pulverized in a domestic grinder at 30ºC to obtain ground rice flour for further analysis.

Sample extraction

After germination for rough rice, the husks of grains were removed before extraction. The extraction for each sample was performed in duplicate. The method of Jang and Xu (2009) with slight modification was used for extraction step. Briefly, one gram of each sample was transferred into a test tube (25 x 150 mm) to which methanol (3 mL) was added, and the mixture was vortex mixed for 30 sec. The test tubes were capped and placed in a 60°C water bath for 20 min. These test tubes were vortex mixed twice during the incubation. Then, the methanol layer in each tube was separated by centrifugation at 10,000 rpm for 10 min. The solvent supernatant was transferred to a 10 mL volumetric flask. The residue was again mixed with 3 mL of methanol. The supernatant was separated as previously described and combined with the previous supernatant. The tube containing supernatant was adjusted to 10 mL. The extracted solution was kept in 0°C until analysis.

Determination of total anthocyanin content

The total anthocyanin content (TAC) was determined by the pH-differential method (Giusti and Wrolstad, 2001). Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5. The pH-differential method is based on this reaction, and permits accurate and rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds.

Briefly, transfer 1 mL extracted solution into 10 mL volumetric flask for preparing two dilutions of the sample, one adjust volume with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each. Let these dilutions equilibrate for 15 min. Measure the absorbance of each dilution at the 510 and 700 nm (to correct for haze), against a blank cell filled with distilled water. All measurements should be made between 15 min and 1 hr after sample preparation, since longer standing times tend to increase observed readings. Absorbance readings are made against water blanks. The samples to be measured should be clear and contain no haze or sediments; however, some colloidal materials may be suspended in the sample, causing scattering of light and a cloudy appearance (haze). This scattering of light needs to be corrected for by reading at a wavelength where no absorbance of the sample occurs, i.e., 700 nm. Calculate the absorbance of the diluted sample (A) as follows:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon x1)$$

and it was converted to mg of total anthocyanin content /100 g sample.

Where MW is the molecular weight , DF is the dilution factor, and ε is the molar absorptivity, calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 and ε= 26,900

Determination of Trolox equivalent antioxidant capacity

The Trolox equivalent antioxidant capacity (TEAC) was performed using the DPPH Free Radical Scavenging Method (Yue and Xu, 2008). The DPPH reagent (0.025 g) was dissolved in 1000 mL of

methanol for preparing the DPPH reagent solution. The bran extract solution which was reconstituted with 6.0 mL of methanol for being employed to measure TPC was used for the DPPH free radical scavenging test. Two milliliters of DPPH solution was mixed with 50, 100 and 150 µL of the extract/methanol solution and transferred to a spectrophotometer cuvette. The reaction solution was carried out at 25°C for 30 min in a dark room. Then the absorbance of the reaction mixture was monitored at 515 nm using a UV-visible spectrophotometer. The inhibition percentage of the absorbance of the DPPH solution was calculated using the following equations:

$$\text{Inhibition \%} = [(\text{Abs } t_{0\ min} - \text{Abs } t_{30\ min})/\text{Abs } t_{0\ min} \times 100$$

Where Abs $t_{0\ min}$ was the absorbance of DPPH at zero time and Abs $t_{30\ min}$ the absorbance of DPPH after 30 min of incubation for the reaction.

The inhibition percentage of the absorbance of DPPH was plotted against each quantity of the extract solution to obtain a regression line. Trolox (0.5 mM) in methanol was used as a standard to convert the inhibition capability of the extract solution to the trolox equivalent antioxidant activity. The ratio of the slopes of the regression lines of the extract solution and the Trolox solution was defined as the trolox equivalent antioxidant capacity. Then, it was converted to µmol of trolox equivalent (TE)/g of rice.

Determination of ferric reducing antioxidant power (FRAP)

The FRAP was performed according to methods described by Benzie and Strain (1999). Briefly, freshly prepared FRAP reagent consisted of 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:1:1 (v/v/v). The 200 µL of rice extract was mixed with 1.3 mL of the FRAP reagent and after 30 min of incubation at 37°C, absorption was measured at 595 using a spectrophotometer. Aqueous or methanolic solutions of known Fe(II) concentration are used for calibration of the FRAP assay. FRAP values, expressed as mmol of Fe(II) equivalent per g rice, was obtained by comparing the absorption change in the test mixture with doses obtained from Fe(II) standard concentrations curve.

Results

The germination percentage of rice at the different germination time

The germination percentage of rice at the different germination time is shown in Figure 1. When the germination time increase, the germination percentage increase. The prime objective of germination is to promote the development of hydrolytic enzymes that are inactive in raw seeds (Ayernor and Ocloo, 2007). At different germination stage, rice grains undergo different biochemical changes. Changes in germination rate, reducing sugars, free amino acids, soluble protein and total protein, and activities of amylase and protease during 5 days of germination was investigated by Veluppillai *et al*. (2009) and found that free amino acids and soluble protein contents increased on the 5th day of germination of rice grains grown in Sri Lanka. However, the germination time in this study was limited at 24 hrs to prevent microbial contamination.

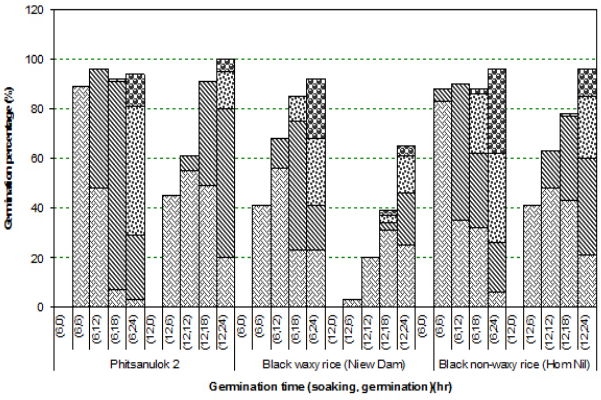

Figure 1. Germination percentage of rice at the different germination time

Stage 1: germ of rice was germinated not more than 1 mm.
Stage 2: germ of rice was germinated more than 1 mm. and the root was not appeared.
Stage 3: germ of rice was germinated more than 1 mm. and the root appeared < 3 mm.
Stage 4: germ of rice was germinated more than 1 mm and the root appeared > 3 mm.

A useful depiction of the progress of germination was provided by Nonogaki *et al.* (2010). The time course of water uptake by a germinating seed was divided into 3 phases. In phase I, there is rapid imbibition of water by the dry seed until all of the matrices and cell contents are fully hydrated, followed by a period of limited water uptake (Phase II). The increase in water uptake associated with Phase III is initially, and briefly, related to completion of germination. The slight increase in water content is followed by a much larger uptake as the cells of the growing radical. In this experiment, the growing radical was visually observed in stage 3. From Figure 1, it can be seen that germination percentage of dehulled rice was higher than that of rough rice. This was due to the husk of grains which act as a barrier for water uptake into kernels. The husk of black waxy rice (Niew Dam) is the thickest amongst three samples, resulting in the lowest germination percentage.

Effect of germination on TAC of different rice varieties

The TAC of ungerminated and germinated rice is shown in Figure 2. For control (ungerminated rice) samples, TAC content of Phitsanulok 2, Niew Dam, and Hom Nil rice was 3.26, 103.45, and 9.79 mg/100 g. of rice, respectively. It can be seen that Niew Dam rice contains the highest amount of naturally occurring anthocyanin among three varieties, which is agreeable to those reported (109 mg/100 g rice) by Sompong *et al*. (2011). Many studies have reported that black rice is more abundant in anthocyanin and other phenolic compounds compared to that of white rice (Rye *et al*., 1998; Zhang *et al*., 2006). These phytochemical compounds usually accumulated in pericarp or bran of rice kernels. These compounds are pigmented- containing related to unique colors such as purple, red, or black. Black and red rice contain 2 main compounds of anthocyanin; cyanidin 3-glucoside (C3G) and peonidin 3-glucoside (P3G), as reported by several researchers (Abdel-Aal *et al*., 2006; Rye *et al*., 1998; Sompong *et al*., 2011), in which C3G comprises approximately 93% of the quantified anthocyanin. According to the findings by Jittorntrum *et al*. (2009), rice bran extracts of dark violet grain called "riceberry", a Thai cross-bred between Hom Nil Rice and Thai Jasmine/ Fragrant Rice or Khao Dawk Mali 105 contained major and minor proanthocyanidin in rice as refer to cyaniding and peonidin in the amount of 150.81 and 66.76 mg/100g, respectively. These generally found pigment of fruits, vegetables, and colored rice have important roles in reducing risk of cancer and other chronic diseases because of their free radicals scavenging capacities (Wang and Stoner, 2008; Shih *et al*., 2007; Elisia *et al*., 2007; Elisia and Kitts, 2008) .

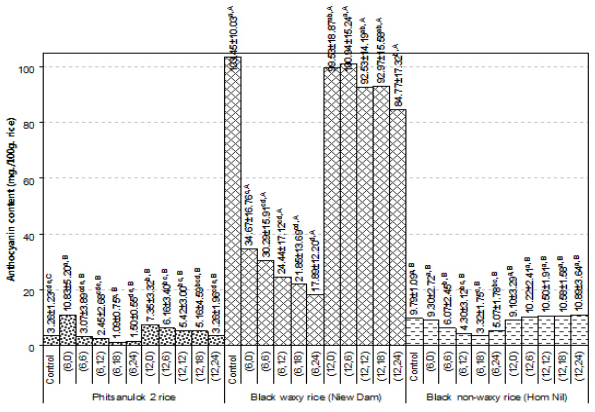

Figure 2. The anthocyanin content of different rice varieties and germination conditions

In parenthesis, the first and second numbers indicate soaking times and germination times (hrs.), respectively

Different small letters (a,b,c) indicate statistically different anthocyanin content within the same variety ($p \leq 0.05$) by DMRT

Different capital letters (A,B,C) indicate statistically different anthocyanin content between varieties of rice germinated in the same condition ($p \leq 0.05$) by DMRT

Similarly, in a comparison among varieties after germination, it was found that germinated black waxy rice (Niew Dam) showed the highest TAC compared to other samples. The amount of anthocyanin content of germinated Phitsanulok 2 ,Niew Dam, and Hom Nil rice ranged between 1.09-10.83 mg/100 g of rice, 17.89-99.53 mg/100 g of rice, and 3.32-1.89 mg/100 g of rice, respectively.

After germination, it was shown that for all rice varieties, germination had more effect on dehulled rice (soaking time 6 hrs) compared to rough rice (soaking time 12 hrs) It can be been seen that germinated rice prepared from rough rice retained higher levels of anthocyanin content, which was not significantly different compared to those of control. This was due to protection of anthocyanin loss by the husk during soaking stage. The husk plays important role in inhibiting exposure of rice kernels to air and light, which causes oxidation to take place. Taylor and Briggs (1990) reported that accumulation of anthocyanin in plant is controlled by multiple regulatory genes and induced by various factors such as light. Pasko *et al*. (2009), who studied anthocyanin content in amaranth and quinoa seeds and sprouts during their growth, reported that dark grown plant generally accumulates fewer anthocyanin as compared to light grown plant. In this study, rice grains were kept in dark during germination, which could partially explain that TAC in rice grains did not increase as germination time increased. Results of the study conducted by Moongngarm and Saetung (2010) revealed that germinated rough rice contained significantly higher amount of bioactive compounds and other nutrients such as α-tocopherol, γ- oryzanol, thiamine, niacin, and pyridoxine compared to those of germinated brown rice and ungerminated rice. Preparation of germinated rice using rough rice as raw material was also advantageous in terms of economy and germination operation as reported by Moongngarm and Saetung (2010). Hence, in order to maintain bioactive compounds such as anthocyanin in finished product, rough rice would be a more suitable form for production of germinated –colored rice.

Effect of germination on antioxidant activity of different rice varieties

The effect of germination on TEAC and FRAP of different rice varieties before and after germination is shown in Figure 3 and Figure 4, respectively. The results of TEAC and FRAP was agreeable in which the Niew Dam rice showed higher antioxidant activity than the other samples. The average values are 4.68, 10.11 and 7.92 µmol Trolox equiv./g rice, and 4.56, 7.20, and 6.32 mmol of Fe(II) equiv./g rice

for germinated Phitsanulok 2, Niew Dam and Hom Nil, respectively. However, it can be seen amongst 3 rice varieties in the present study that antioxidant activity values determined by FRAP method were not distinctive as those found by TEAC, which could be due to different mechanisms involved. Trolox equivalent antioxidant capacity has become routine practice in evaluating antioxidant activity of plant material due to its simplicity of the assay and the fact that it can be used in aqueous and lipid phases (Sompong *et al.*, 2011). On the contrary, the principle of FRAP assay is based on the reduction of the Fe(III)-TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 595 nm (Benzie and Strain, 1999).

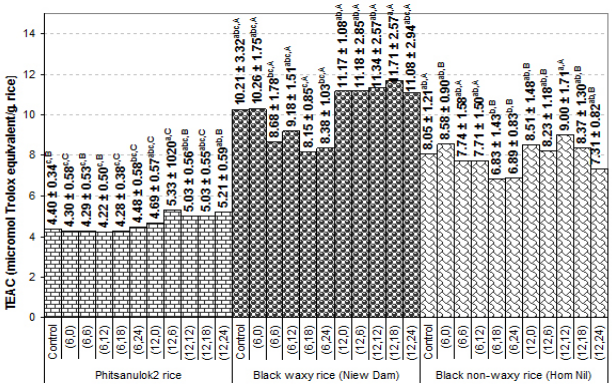

Figure 3. Trolox equivalent antioxidant capacity (TEAC) of different rice varieties and germination conditions

In parenthesis, the first and second numbers indicate soaking times and germination times (hrs.), respectively
Different small letters (a,b,c) indicate statistically different TEAC within the same variety ($p \leq 0.05$) by DMRT
Different capital letters (A,B,C) indicate statistically different TEAC between varieties of rice germinated in the same condition ($p \leq 0.05$) by DMRT

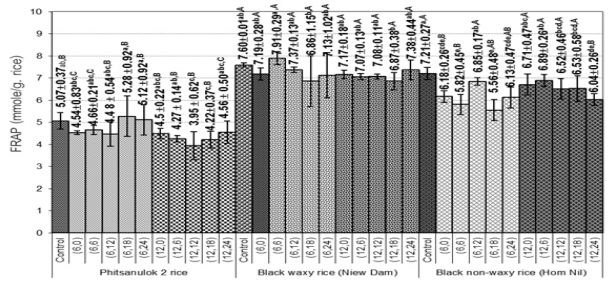

Figure 4. Ferric reducing antioxidant power (FRAP) of different rice varieties and germination conditions

In parenthesis, the first and second numbers indicate soaking times and germination times (hrs.), respectively
Different small letters (a,b,c) indicate statistically different TEAC within the same variety ($p \leq 0.05$) by DMRT
Different capital letters (A,B,C) indicate statistically different TEAC between varieties of rice germinated in the same condition ($p \leq 0.05$) by DMRT

From Figure 3, it can be seen that germination had no effect on TEAC of germinated colored rice prepared from rough rice. However, TEAC of germinated colored rice that prepared from dehulled rice was slightly decreased when the germination time increased. On the other hand, regarding non-pigmented variety (Phitsanulok 2), a germination process was able to increase TEAC when rough rice was used as a raw material. This may partially due to changes of other antioxidants in rice grains during germination. Regular brown rice is normally contains beneficial compounds which also act as antioxidants such as γ- oryzanol and tocopherol. The increased amount of tocopherol in germinated rice was reported by Jiamyangyuen and Ooraikul (2009). Tocopherol is more abundant in seeds than in any other plant tissues (Mansfield and Braiarty, 1992) It is a lipophillic antioxidant synthesized by all plants and plays an important role in limiting non-enzymatic lipid oxidation during seed storage, germination, and early seedling development (Sattler *et al*., 2004). During germination, various reactive oxygen species (ROS) are generated as by-products of metabolism. This group of ROS includes superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide radicals ($H_2O_2^{\cdot-}$), and hydroxyl radicals (OH^-). The formation of these oxygen radicals results in the accumulation of lipid hydroperoxides by radical chain oxidation via phospholipids peroxy radicals within membranes. Therefore, it was hypothesized that this could be related to the increase of antioxidant activity in large unilamellar vesicles observed in germinated seeds (Frias *et al*., 2004).

To describe the relationship between antioxidant content and activity, the correlation study was performed and reported as correlation coefficient (r). It showed that positive correlations between TAC and TEAC were found for pigmented rice varieties (r=0.79 and r=0.86 for Niew Dam and Hom Nil, respectively), indicating that anthocyanins play an important role as they are responsible for the antioxidant activity in the pigmented-germinated rice samples.

Conclusions

Rice containing pigments is one of good sources of antioxidant compounds, including anthocyanin. As far as antioxidant and their activity are concerned, it was found that colored rice contains more anthocyanin and antioxidant activity than non-colored rice. In addition, after germination, rough rice retained higher levels of anthocyanin and antioxidant activity than that of rice prepared from dehulled. Therefore, rice with husk intact should be employed for the preparation of germinated pigmented rice to protect anthocyanin and its antioxidant activity loss during germination process. It is necessary to conduct additional studies to further investigate which specific environmental factors such as temperature, soil, and water that influence the anthocyanin contents of color rice seed and sprouts, which could help maximize anthocyanin

production.

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