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Effective Antioxidant Activities of Anthocyanins as Affected by pH of Antioxidant Assays

Paradorn NGAMDEE[1,*] and Sudarat JIAMYANGYUEN[2]

[1]*Department of Food Science and Technology, Faculty of Science and Technology, Thepsatri Rajabhat University, Lopburi 15000, Thailand*
[2]*Department of Agro-Industry, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok 65000, Thailand*

([*]Corresponding author's e-mail: nparadornn@gmail.com)

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Abstract

This study analyzed the effect of pH on antioxidant activities of anthocyanins. Five black glutinous rice bran crude extracts (BBCs) with different anthocyanin content were compared. They were referred to as Moo Ser (MS), Saked (SK), Phayao (PY), Phichit (PC) and Kao Kor (KK). The antioxidant activities were evaluated using 2 *in vitro* antioxidant assays conducted at different pH, DPPH (pH 5.5) and FRAP (pH 3.6). Total anthocyanin content (TAC) of KK (23 g/kg) was the highest, followed by PC (13 g/kg), PY and SK (both 11 g/kg), and MS (10 g/kg). The pH of the assays affected the activity of the anthocyanins as the KK showed DPPH scavenging activity comparable to other BBCs but exhibited significantly greater FRAP antioxidant activity. To eliminate possible interference by other active components in the BBC, anthocyanin-rich isolate (ARI) was then isolated from KK. The ARI contained 90 % cyanidin-3-glucoside as analyzed by HPLC. The ARI was dissolved in 5 different buffer solutions (pH 1, 3, 5, 7, and 9) and incubated for 1 h, 3 days, and 1 week before the evaluation of antioxidant activities. All ARI-containing buffer solutions exhibited comparable DPPH scavenging activity. However, the ones at pH 5 and 7 solutions were less reactive than others when tested with the FRAP assay. These results indicated that anthocyanin is a good antioxidant not only in an acidic environment but also in higher pH ranges. This suggests that the utilization of anthocyanins as natural antioxidants in products such as food and cosmetics is not limited by the pH of the products.

Keywords: Anthocyanins, pH, Antioxidant, DPPH, FRAP

Introduction

Anthocyanins are known as good antioxidants that show a number of health benefits including relief oxidative stress, prevention of cardiovascular diseases, anti-inflammatory activity, anti-carcinogenic activity, prevention of obesity, control of diabetes, improvement of eye vision, and antimicrobial activity [1]. Anthocyanins are found in parts of plants: tubers, stems, leafs, flowers, fruits, and seeds. Some cereal grains, such as wheat, barley, corn, and rice, were reported to have as high as 3.2 mg/g of total anthocyanin content (TAC) [2]. Among these grains, black pigmented rice is one of the most studied grain since it has high TAC (approximately 3.5 mg/g dry weight (DW) in whole grain rice and 12.5 mg/g DW in rice bran) [3].

Black pigmented rice or black rice (*Oryza sativa* L. *indica*), both glutinous and non-glutinous cultivars, are consumed in Thailand. Plaitho *et al.* [4] reported that Thai black glutinous rice has approximately 100 times higher TAC than that of the non-glutinous one. Two major anthocyanins in black rice are cyanidin-3-*O*-glucoside (Cy3G) and peonidin-3-*O*-glucoside (Pn3G), which their contents accounted for approximately 51 - 84 and 6 - 16 %, respectively, of all anthocyanins in the black rice [3].

A number of studies were conducted on the antioxidant activity of anthocyanin-rich crude extracts obtained from various plants. Among these studies, *in vitro* antioxidant assays are commonly used to evaluate the potential health benefits of the crude extracts. The examples of these antioxidant assays are oxygen radical absorbance capacity (ORAC), Folin-Ciocalteu reagent (FCR), trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity [5]. The interesting aspect is that most of these *in vitro* antioxidant assays are performed in neutral pH environments. However, the FRAP assay is conducted in the acetate buffer solution pH 3.6 [5]. An anthocyanin acts as an antioxidant by donating a phenolic hydrogen atom to a free radical [6]. It has been known that anthocyanins are extremely susceptible to the change of the pH and their chemical structures are stable only in a highly acidic environment [6]. Due to this fact, the anthocyanins in an alkaline environment should lose their ability to donate proton since their active structures, flavylium cation, are altered by the higher pH.

In the solution with pH higher than 2, the structure of an anthocyanin is changed or even degraded into different forms depending on the pH and anthocyanin derivatives [6]. Cevallos-Casals and Cisneros-Zevallos [7] reported that the half-life of anthocyanins from a plant crude extract in a pH 0.9 solution was longer than 138 days; however, the half-life was decreased to be less than 1 h as the pH increased to 3 and higher. According to these reports, anthocyanins should not be directly involved in the antioxidant activity result of an anthocyanin-containing crude extract that is tested with an antioxidant assay performed in a high pH solution.

This notion agrees with the finding of Zhang *et al.* [8] who showed that ORAC antioxidant activity of an anthocyanin-rich crude extract was weakly correlated with the anthocyanin content. The decreasing in antioxidant capacity of anthocyanins by the pH has become more obvious when the antioxidant activity of the anthocyanins was compared between the FRAP and the DPPH assays. According to the report of Sompong *et al.* [9], the antioxidant activity of the anthocyanin-rich black rice crude extract tested with the FRAP assay increased with the increase of the TAC value, whereas the DPPH scavenging activity result of the crude extract was not correlated with the TAC. This finding indicates that anthocyanins could play a role as the strong antioxidants only in the acidic environment (the FRAP assays is performed in a pH 3.6 buffer solution). However, additional evidence is needed in order to assure that the antioxidant activity of anthocyanins is affected by the pH of the antioxidant assays. An important reason is that it is highly possible that crude plant extracts could also contain other bioactive compounds, presumably phenolic acids and proanthocyanidins [3], which could lead to the incorrect interpretation of the antioxidant activity result of the anthocyanins.

This study used simple experimental procedures to reveal a significant scientific evidence of the antioxidant activity study of the anthocyanins. Black glutinous rice bran samples containing different anthocyanin content were analyzed for their antioxidant activities using 2 electron transfer-based antioxidant assays, the DPPH and FRAP assays. Anthocyanins were isolated from a black glutinous rice bran crude extract and used for the tests in order to eliminate possible interference of other bioactive compounds that could mislead the interpretation of the antioxidant results. The effects of the pH of the solutions with different pH were used to dissolve anthocyanins and the storage time were also measured.

Materials and methods

Chemicals

Chemicals for antioxidant assays were 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Fluka, Germany), 2,4,6-tripyridyl-s-triazine (TPTZ, Sigma-Aldrich, USA), ferric chloride hexahydrate ($Fe.Cl_3.6H_2O$), and Trolox (Sigma, USA). Analytical grade chemicals for buffer preparation: potassium chloride (KCl), sodium hydroxide (NaOH), sodium acetate tri-hydrate ($CH_3CO_2Na.3H_2O$), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from LOBA Chemie, India, and sodium tetraborate ($Na_2B_4O_7$) was purchased from Sigma-Aldrich, USA. Glacial acetic acid, hydrochloric acid, and HPLC grade mobile phase (water and acetonitrile) were purchased from RCI Labscan, Thailand. Solvents used in the experiment were analytical grade. Standard anthocyanins, cyanidin-3-glucoside chloride (Cy3G) and peonidin-3-glucoside chloride (Pn3G), were purchased from Sigma, USA.

Rice samples and samples preparation

Five black glutinous rice cultivars, Moo Ser (MS), Saked (SK), Phayao (PY), Phichit (PC) and Kao Kor (KK), were purchased from farmers from the north of Thailand. Paddy rice (1 kg) was dehulled and subsequently milled to obtain rice bran powder using a small electric rice miller (NW1000 TURBO, Natrawee Technology Co., Ltd.). Rice bran was stored in an aluminum foil vacuum-sealed bag and kept at –20 ± 2 °C for further analysis.

Extraction of anthocyanins

Black glutinous rice bran powder was defatted by stirring with hexane and dried. Five grams of rice bran powder was extracted with 30 mL of 0.15 N HCl in methanol and stirred for 1 h. The solid fraction was separated by centrifugation at 6000 rpm for 15 min at 25 °C. Three more extractions were repeated with the solid fraction using the same procedure. The supernatants from 4 extractions were combined and the solvent was removed using a rotary evaporator at 40 °C. The anthocyanin-rich black glutinous rice bran crude extract (BBC) was obtained and stored at –20 ± 2 °C for further analysis.

Determination of total anthocyanin content

TAC of BBC was determined using the pH differential method [10]. Briefly, 0.1 mL of sample was diluted in separate buffer solutions (3 mL) at pH 1.0 and 4.5 and the absorbance at 510 and 700 nm were measured using a UV-visible spectrophotometer (HACH DR-4000U, USA). The absorbance values were calculated using the following equation;

$$\text{Absorbance } (A) = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}} \quad (1)$$

where A_{510} is the absorbance at 510 nm and A_{700} is the absorbance at 700 nm. The obtained A value was used to calculate for TAC using the following equation;

$$\text{TAC (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1) \quad (2)$$

where A is the absorbance obtained from the first equation, MW is the molecular weight of cyanidin-3-glucoside (449.2), DF is dilution coefficient (3 mL/0.1 mL = 30), and ε molar absorptivity of cyanidin-3-glucoside (26,900). The result was expressed as g/kg DW.

Isolation of anthocyanins

Anthocyanins were isolated from BBC using column chromatography. Firstly, BBC was partially purified with Amberlite XAD-7 (20 - 60 mesh, Sigma-Aldrich) in order to enrich the anthocyanins content and eliminate other hydrophilic compounds [11]. The Amberlite XAD-7 resin (50 g) was soaked with distilled water overnight and packed in a glass column (Ø2.5×30 cm). The packed column was washed with 200 mL distilled water. BBC was dissolved in distilled water and loaded into the column. The BBC was washed with distilled water in order to eliminate water-soluble molecules while anthocyanins and other phenolics were absorbed on the resin. Acidified methanol (0.15 M HCl) was used to elute the compounds that were absorbed on the resin. This partially purified BBC was concentrated using a rotary evaporator at 40 ± 1 °C.

Secondly, the concentrate solution of the partially purified BBC was partitioned using Sephadex LH-20 column (25 - 100 μm particle size) [12]. The resin was soaked with 20 % methanol overnight before loading into a glass column (Ø2.5×30 cm). The concentrate partially purified BBC (1 mL) was loaded into the column and eluted with 200 mL of 20 % methanol. The anthocyanin-rich isolate (ARI) fraction was obtained by eluting the column with 200 mL of 70 % methanol. Every 5 mL of the eluent was collected as a fraction and determined for the absorbance at 510 and 280 nm using a spectrophotometer. The fractions showed the absorbance at 510 nm and was collected as anthocyanin fractions. The column was cleaned with 100 mL of 90 % methanol and 200 mL of 70 % acetone, respectively. All anthocyanins containing fractions were combined and the solvent was evaporated using

a rotary evaporator at 40 ± 1 °C. The ARI was freeze-dried to obtain dry powder and stored at −40 ± 2 °C for further analysis.

HPLC analysis

The BBC and ARI were analyzed using HPLC-PDA (LC-10AD VP, Shimadzu, Japan) equipped with reversed phase C-18 HPLC column (Inertsil® ODS-3, 4.6×250 mm^2, 5 μm). The mobile phases were 0.1 % formic acid (A) and 0.1 % formic acid in acetonitrile (B). The injection volume was 100 μL. The separation was performed at 1 mL/min with step gradient elution program as follows: 0 - 5 min; 0 - 8 % B, 5 - 15 min; 20 % B, 15 - 20 min; 50 % B, 20 - 21 min; 90 % B, 21 - 25 min; 90 % B, 25 - 26 min; 0 % B, and 26 - 30 min, 100 % A. The absorbance at 280 nm was used to monitor the separation of anthocyanins by comparing to the corresponding authentic standards.

Preparation of buffer solutions

Buffer solution pH 1 was prepared by adjusting the pH of 0.1 M KCl with 0.1 M HCl to the desired pH. Acetate buffer solutions, pH 3 and 5, were prepared by adjusting the pH of 0.1 M acetic acid with 0.1 M sodium acetate to the desired pH. Phosphate buffer solution pH 7 was prepared by adjusting the pH of 0.1 M potassium dihydrogen phosphate with 0.1 M NaOH. Borax buffer pH 9 was prepared by adjusting the pH of 0.025 M Borax with 0.1 M HCl.

DPPH scavenging activity

The pH controlled antioxidant activity against DPPH free radical of the BBC and the ARI was analyzed using the method described by Sharma and Bhat [13]. The sample was diluted with 0.1 N HCl in methanol and used for the test. DPPH solution (0.1 mM) was prepared in methanol buffered with acetate buffer (pH 5.5) (prepare by mixing 40 mL of 0.1 M acetate buffer (pH 5.5) with 60 mL methanol). The sample solution (30 uL) was mixed with the DPPH solution (3 mL) and let react for 30 min at room temperature (30 ± 1 °C) in the dark. The absorbance of the mixture was determined at 517 nm. Standard plot of Trolox at 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/mL was performed. The result was expressed as milligram Trolox equivalent (TE)/100 g DW and calculated from the Trolox standard plot.

Ferric reducing antioxidant power (FRAP) antioxidant activity

Scavenging activity of BBC and ARI using FRAP assay were analyzed using the method described by Benzie and Strain [14]. Sample solutions were prepared and diluted with 0.1 N HCl in methanol. The sample solution (30 uL) was mixed with 3 mL of FRAP reagent (10 parts of 0.3 M acetate buffer solution pH 3.6, 1 part of 10 mM TPTZ solution, and 1 part of 20 mM $Fe.Cl_3.6H_2O$ solution). After the mixture was kept at room temperature (30 ± 1 °C) in the dark for 30 min, the absorbance was measured at 595 nm using a UV-visible spectrophotometer. The activity of the standard Trolox at 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/mL was plotted. The result was expressed as mg TE/100 g DW.

Antioxidant activities of the anthocyanins in different pH environment

The effect of the pH environment on the antioxidant activities of anthocyanins was studied. ARI solution prepared in 0.15 N HCl methanol was diluted to the same concentration at 0.4 mg/mL in different buffer solutions pH 1, 3, 5, 7, and 9. After these solutions were stored for 1 h, 3 days, and 1 week, they were measured for the antioxidant activities by using DPPH and FRAP assays. The control experiment (the buffer solutions are used instead of the sample solution) were performed in order to evaluate the effect of the buffer solutions on the antioxidant activity results. The experiment was performed with 3 external replications. The mixed solutions were stored in a refrigerator at 4 ± 1 °C during the analysis period as to reduce the effect of temperature on the degradation of anthocyanins [15].

Statistical analysis

Results were expressed as mean values with standard deviation (SD) from at least triplicate experiments. Statistical analysis was performed using SPSS v 16.0 (SPSS Inc., Chicago, Illinois, USA), and statistical significance was assessed using ANOVA followed by Duncan at 95 % confidence level ($P \leq 0.05$). Bivariate correlation was used for the analysis of the correlation and the result was represented as Pearson's correlation coefficient (r).

Results and discussion

Total anthocyanin content (TAC)

The anthocyanin content of the BBCs from the bran of 5 black glutinous rice cultivars is shown in **Table 1**. The TAC value of KK (23 g/kg DW) was significantly higher ($P \leq 0.05$) than those of the other rice cultivars (which ranged from 10 to 13 g/kg DW). The PC showed the second highest TAC, followed by SK and PY, the 2 of which did not have statistically different TAC values. The MS contained the lowest TAC. Anthocyanin content can vary due to a number of factors, such as cultivation method, soil and light conditions, other growing conditions, and especially the rice cultivars themselves [8]. The bran of black glutinous rice contained TAC ranging from 23 g/kg DW in *Oryza sativa* L. *japonica* to 12 - 34 g/kg DW in *Oryza sativa* L. *indica* cultivars [8].

Antioxidant activities

Antioxidant activities of BBC from the 5 black glutinous rice cultivars against free radicals were analyzed using DPPH and FRAP assays, and the results are presented in **Table 1**. These 2 assays use the same reaction mechanism, electron-transfer reaction, to determine the antioxidant activity of active compounds, which eliminates any possible doubt over the effect of the reaction mechanism itself. The DPPH scavenging activities of KK (749 mg TE/100 g DW) and SK (683 mg TE/100 g DW) were the highest, followed by those of PC (631 mg TE/100 g DW), PY (589 mg TE/100 g DW), and MS (561 mg TE/100 g DW). The finding that KK and SK displayed equivalent DPPH activity in spite of KK having a TAC 2.3 times higher than that of SK, suggested that anthocyanins may not be the predominant scavengers against the DPPH free radical. The reason that anthocyanins did not show as much activity as expected could be that they were degraded due to the pH of the test solution (pH 5.5), resulting in a reduced ability of the anthocyanins to donate their proton [16]. This notion would agree with reports indicating that the anthocyanin content of a pH 5 solution was reduced by more than 80 % within 1 h [7].

The above assumption was supported by the FRAP assay results. The KK (125 mg TE/100 g DW) exhibited significantly ($P \leq 0.05$) greater FRAP inhibition than that of PC, PY, SK, or MS, the 4 of which amongst themselves displayed statistically equivalent ($P > 0.05$) activity levels ranging from 38 to 48 mg TE/100 g DW. The FRAP activity of KK was 2.6 times higher than that of the other BBCs. This ratio was corresponded closely to the ratio of TAC values (2.3 times) mentioned above. Anthocyanins tend to maintain their active structure in the FRAP assay for a longer time than in the DPPH assay, as the former is conducted in a buffer solution of pH 3.6 (compared to the DPPH buffer solution pH of 5.5). Although anthocyanins are stable only at pH 1 at room temperature [16], anthocyanins from some sources are able to maintain 50 % of their content after 1 h in a pH 4 environment [7].

The correlation between TAC and DPPH and FRAP activities is shown in **Table 1**. Both DPPH and FRAP activities were significantly positively correlated with the anthocyanin content. However, r values indicated that the FRAP assay (r = 0.981) was more correlated with TAC than the DPPH assay (r = 0.678). This result further supports the assumption that pH of the assay solution affects the antioxidant power of anthocyanins.

However, further evidence is needed in order to definitively conclude that these results are mainly caused by the pH of the antioxidant assay solutions. One reason is that BBCs may contain other bioactive compounds that could interfere with the antioxidant activity of anthocyanins. Hence the isolation of anthocyanins from BBC was performed.

Table 1 Total anthocyanin content (TAC), antioxidant activities of black glutinous rice bran crude extracts (BBCs) and the correlation between antioxidant activities and TAC.

BBC	TAC (g/kg DW)	Antioxidant activity (mg TE/100 g DW)	
		DPPH	FRAP
MS	10 ± 0.2^{d}	561 ± 12^{d}	38 ± 1^{b}
SK	11 ± 0.1^{c}	683 ± 22^{a}	45 ± 9^{b}
PY	11 ± 0.3^{c}	589 ± 25^{c}	47 ± 6^{b}
PC	13 ± 0.5^{b}	631 ± 11^{b}	48 ± 2^{b}
KK	23 ± 0.1^{a}	749 ± 29^{a}	125 ± 4^{a}
r	-	.981*	.678*

*Different superscript letters indicate significant difference ($P \leq 0.05$) of the data in the same column.
**Pearson correlation coefficient (*r*) is significant at 0.01 level (2-tailed).

Anthocyanin-rich isolate (ARI)

The methanolic crude extracts of black rice contain various types of bioactive compounds including phenolic acids and proanthocyanins as reported by Goufo *et al.* [3]. Some phenolic acids, such as ferulic acid, high amounts of which are found in rice [17], exhibit strong antioxidant activity that could be wrongly attributed to anthocyanins [18]. For this reason, in order to eliminate the possible interference of other bioactive compounds, anthocyanins were isolated from BBC using Sephadex LH-20 resin column chromatography. The anthocyanin fraction thus obtained was labeled as anthocyanin-rich isolate (ARI). The KK extract was selected for the isolation as it contains the highest TAC. The absorbance resolutions of fractions from the BBC of KK are shown in **Figure 1**. Most organic compounds, including anthocyanins, absorb the wavelength at 280 nm, however only anthocyanins show absorbance at 510 nm. For this reason, the fractions were measured for absorbance at 510 nm and 280 nm in order to monitor the separation and removal of other organic compounds from the BBC, leaving only anthocyanins. In **Figure 1**, the absorbance at 280 nm shows the other organic compounds as fractions 3 to 53, and these were separated by eluting with 20 % methanol. In contrast, the absorbance at 510 nm shows the anthocyanin containing fractions as fractions 56 to 72. These were collected, and the isolate yield was approximately 55 % w/w.

HPLC profiling of anthocyanins

The HPLC profiles of the BBC of KK, ARI, and anthocyanin standards are presented in **Figure 2**. Regarding the chromatogram of anthocyanin standards, the BBC of KK contained Cy3G as a major and Pn3G as a minor anthocyanin, which accounted for approximately 90 and 10 % of total anthocyanins, respectively. The appearance of minor peaks in the KK indicated the presence of other, unwanted compounds, which could be phenolic acids, since they were found in the methanolic crude extract of black rice [17].

In order to eliminate possible interference by unwanted compounds in the BBC, ARI was successfully isolated from the BBC of KK using Sephadex LH-20 column chromatography. The HPLC profile of ARI in **Figure 2** indicates that the ARI was free from interfering compounds, since their corresponding peaks found in the BBC of KK were eliminated, while the peaks of Cy3G and Pn3G were still preserved and present.

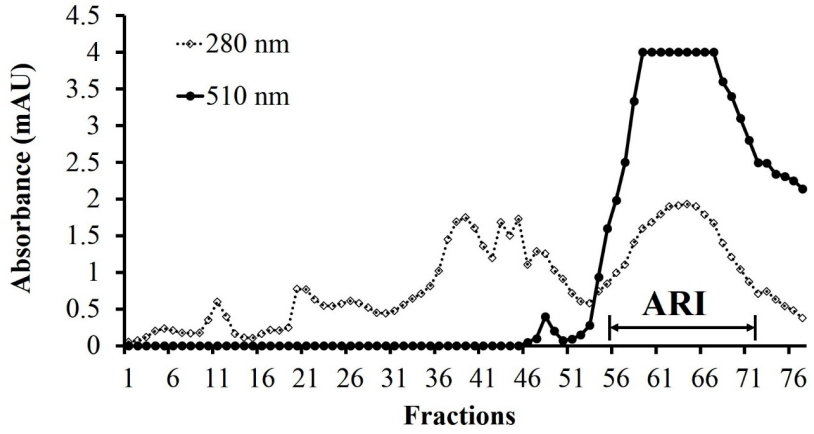

Figure 1 Chromatographic resolution at 280 nm and 510 nm of the black glutinous rice bran crude extract (BBC) as separated by Sephadex LH-20. The ARI label indicates fractions collected as the anthocyanin-rich isolate.

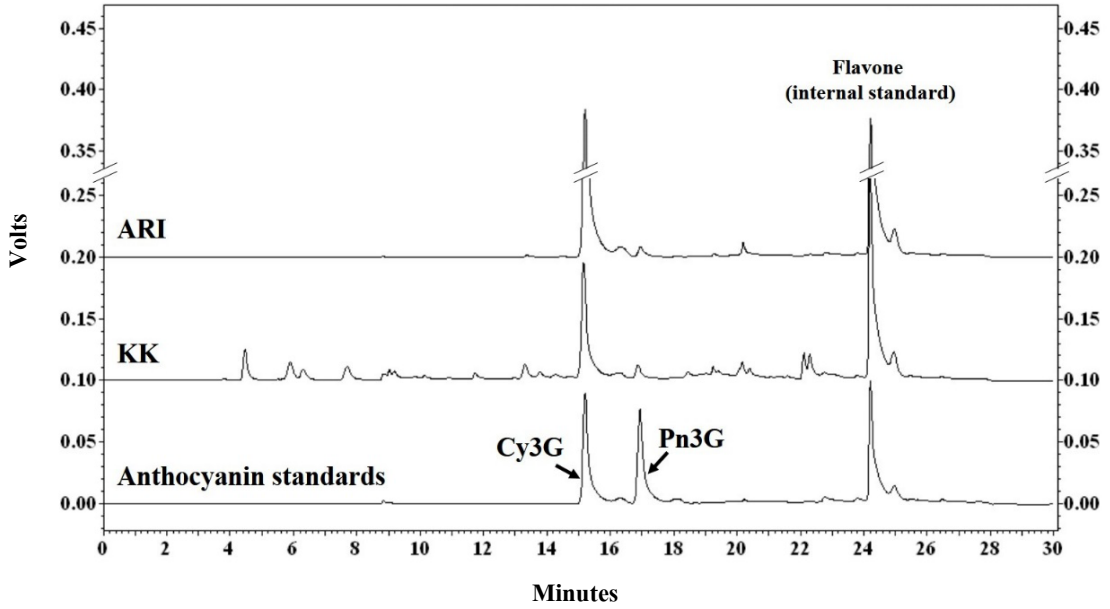

Figure 2 HPLC chromatograms measured at 280 nm of the ARI, black glutinous rice bran crude extract of the KK (KK), and anthocyanin standards.

The effect of pH on antioxidant activity of anthocyanins

ARI was diluted in 5 buffer solutions with different pH (pH = 1, 3, 5, 7, and 9) and the ARI antioxidant activity within each pH was determined with DPPH and FRAP assays. The results are presented in **Table 2**. The ARI-containing buffer solutions were measured for antioxidant activities after 1 h, 3 days, and 1 week of incubation. The control experiments indicated that the buffer solutions had no interference on the antioxidant activities at the standard sample volume of 30 μL used for the evaluation (data not shown).

Due to the fact that anthocyanins are stable at pH 1, ARI in a pH 1 buffer solution should exhibit significantly better antioxidant than the one in other higher pH buffer solutions (pH 3, 5, 7 and 9). However, after 1 h of incubation, the ARI-containing buffer solution pH 1 showed comparable DPPH scavenging activity (127 - 142 mg TE/100 g DW) to that of the higher pH buffer solutions (with the exception of the pH 9 buffer which was significantly lower) (**Table 2**). This result supports the suggestion of Amorati *et al.* [5] who mentioned that the mechanism of the DPPH assay is not the hydrogen transfer but the electron transfer of a phenoxide anion to the DPPH radical. Due to this aspect, although the alkali of the DPPH test solution has changed the chemical structure of an anthocyanin (cause the anthocyanin to lose its ability to donate proton), the phenol groups are still presented on the anthocyanin structure. These phenol groups can form the phenoxide anion and exhibited electron transfer property to counteract with the DPPH radical. This notion was proved by the statistically non-different of the DPPH antioxidant activity results of the ARI incubated in all pH buffer solutions (pH 3, 5, 7 and 9) for 3 days and 1 week. These results ensure the alteration of the anthocyanin structure in the higher pH buffer solution.

The pH degraded products of anthocyanins were proposed. Castañeda-Ovando *et al.* [6] demonstrated that the chemical structure of anthocyanins changed due to different chemical structures and derivatives at different pH ranges. With the increase of the pH, flavylium cation anthocyanins changed to quinoidal bases (pH 2 - 4), carbinol pseudobase (pH 5 - 6), and degraded to their corresponding derivatives (pH > 7) depending on the anthocyanin species. Since Cy3G was a predominant anthocyanin in ARI, the major degraded product of ARI should be protocatechuic acid [19]. Most phenolic acids are strong antioxidants but protocatechuic acid was the exception [20]. This is the reason that the DPPH scavenging activities of ARI in the buffer solutions pH 9 tended to be lower than that of others at the first hour (**Table 2**).

The FRAP antioxidant activity result at the first hour in **Table 2** shows the different trend of the activity as compared to the corresponding result of the DPPH assay. After ARI was incubated in buffer solutions for 1 h, FRAP antioxidant activity tends to decrease with the increase of the buffer pH. The activity result of the ARI in pH 1 (130 mg TE/100 g DW) was the greatest followed by that of the pH 3 (116 mg TE/100 g DW) but the difference was insignificant ($P > 0.05$). The FRAP activity of the pH 3 was higher than that of the pH 5 (113 mg TE/100 g DW) but again it was insignificant ($P > 0.05$). The FRAP activity of the pH 5 was significantly higher than that of the pH 7 (89 TE/100 g DW). Interestingly, the FRAP activity of the pH 9 was statistically comparable to that of the pH 1 and 3. To explain this phenomenon, the fact about the half-life of anthocyanins is adopted. Anthocyanins could maintain their active structure for a longer time in the solutions with low pH [7]. When tested with the DPPH assay, anthocyanins may not show a strong activity as the assays media was a buffer solution pH 5.5, which cause the change of the anthocyanin structure. In contrast, in the FRAP assay that was conducted in a more acidic buffer solution (pH 3.6), anthocyanins could maintain their active structure for an additional time giving better antioxidant activity result. As additionally explained by Amorati *et al*. [20], the phenolic groups of the anthocyanin degraded products were partially ionized in the alcoholic solvent giving the corresponding phenoxide anions, which act as active electron transfer antioxidants. This is the reason that the ARI-containing buffer solutions pH 5 and 7 showed high DPPH scavenging activity. In contrast, since the FRAP assay was performed in a buffer solution pH 3.6, the phenoxide anion was mostly neutralized and became less reactive resulting in low FRAP antioxidant activity in buffer solution pH 5 and 7.

Table 2 Antioxidant activities measured with DPPH and FRAP assays of ARI dissolved in different buffer solutions and measured at different incubation time.

Antioxidant assay	pH of buffer solution	Time of incubation		
		1 h	3 Days	1 Week
DPPH (mg TE/100 g DW)	pH 1	142 ± 5^{aA}	120 ± 10^{ndB}	118 ± 15^{ndB}
	pH 3	127 ± 4^{abND}	123 ± 13^{ndND}	121 ± 14^{ndND}
	pH 5	135 ± 8^{abND}	122 ± 9^{ndND}	123 ± 5^{ndND}
	pH 7	140 ± 5^{abA}	109 ± 11^{ndB}	106 ± 10^{ndB}
	pH 9	124 ± 11^{bND}	125 ± 12^{ndND}	129 ± 4^{ndND}
FRAP (mg TE/100 g DW)	pH 1	130 ± 6^{aA}	117 ± 3^{aB}	113 ± 5^{aB}
	pH 3	116 ± 7^{abND}	102 ± 7^{bND}	100 ± 3^{bcND}
	pH 5	113 ± 8^{bND}	102 ± 6^{bND}	101 ± 1^{bcND}
	pH 7	89 ± 4^{cA}	83 ± 2^{cB}	73 ± 2^{dC}
	pH 9	121 ± 3^{abA}	103 ± 7^{bB}	104 ± 3^{bB}

*Lowercase and uppercase superscript letters indicate significant difference ($P \leq 0.05$) between means in the same column and row, respectively.
**"nd" and "ND" indicate insignificant difference ($P > 0.05$) between means in the same column and row, respectively.

The latter reason also explains about the high activity of ARI in the buffer solution pH 9. In the case of the buffer solution pH 5 and 7, only some phenol groups were ionized to be phenoxide anion due to the lower basicity. In contrast, the higher basicity of the buffer solution pH 9 causes the ionization of almost all of the phenolic groups in an anthocyanin. This resulted in more available phenoxide anion to transfer the electron to react with the ferric ion in the FRAP assay resulting in higher FRAP antioxidant activity of the ARI-containing buffer solution pH 9 than that of the one the buffer solutions pH 5 and 7.

The storage time also affected the antioxidant activities of the anthocyanins. The DPPH antioxidant activity of the ARI-containing buffer solution measured at 3 days (range from 109 (pH 7) to 125 (pH 9) mg TE/100 g DW) and 1 week (range from 106 (pH 7) to 129 (pH 9) mg TE/100 g DW) was insignificantly different; however generally lower than that of the corresponding 1 h. The decreasing trend of the antioxidant activity during storage also occurred with the FRAP assay. The FRAP antioxidant activity of the ARI-containing buffer solution at 3 days (range from 102 (pH 3 and 5) to 117 (pH 1) mg TE/100 g DW) and 1 week (range from 100 (pH 3) to 113 (pH 1) mg TE/100 g DW) were mostly comparable except for that of the one in the buffer solution pH 7 (83 mg TE/100 g DW). The half-life of Cy3G is used to explain this phenomenon as it is a major anthocyanin in the ARI. Hou *et al.* [21] reported that the half-life of Cy3G that is dissolved in pH buffer solution pH 3, 5, 7, and 9, respectively decreases from 12 h to less than 1 h. As the pH increase, anthocyanins are not in their active form (flavylium cation) resulting in a less antioxidant activity of the anthocyanins.

By comparing to the corresponding KK, the DPPH scavenging activity of the ARI-containing buffer solution pH 1 at 1 h (142 mg TE/100 g DW) was approximately 5 times lower than that of the KK (749

mg TE/100 g DW). This indicates that the KK has high possibility to contain active compounds other than anthocyanins. These compounds are believed to be phenolic acids and proanthocyanidins as they are commonly identified in the methanolic extract of black rice [3]. Amorati *et al.* [20] mentioned that the alkali environment of an antioxidant assay enhances the antioxidant activity of phenolic acids. In contrast, the same authors indicated that the phenolic acids, with specific to caffeic acid, are poor antioxidants in an acidic solution. The notion of Amorati *et al.* agrees with the FRAP antioxidant activity result, which the activity of the ARI-containing buffer solution pH 1 at 1 h (130 mg TE/100 g DW) (**Table 2**) closes to that of the KK (125 mg TE/100 g DW) (**Table 1**).

Conclusions

Two electron transfer-based antioxidant assays, DPPH and FRAP, revealed that the antioxidant activity of the anthocyanins was affected by the pH of the test solution. A suspect was noticed as the high-anthocyanin KK extract showed comparable DPPH antioxidant activity to that of other extracts (had lower anthocyanin content) but it showed considerable higher FRAP antioxidant activity. Anthocyanins were purified from the KK in order to eliminate any possible interference compounds and the ARI was obtained. The HPLC chromatogram indicated that the ARI was mainly anthocyanins. The ARI was dissolved in buffer solutions pH 1, 3, 5, 7, and 9 and these solutions were kept for 1 h, 3 days, and 1 week before they were measured for the antioxidant activities using the DPPH and FRAP assays. The result indicated that the acidic solution used in FRAP assay helps stabilize the active flavylium cation structure of the anthocyanins giving a better antioxidant result than that of the DPPH test, which was conducted in a high pH solution. The DPPH antioxidant activity of the ARI tended to decrease with the increase of the solution pH and the storage time tended to have less effect on the DPPH antioxidant activity of the ARI. The acidic solution used in the FRAP assay had some effect on the antioxidant activity of the ARI dissolved in different pH buffer solutions and stored with different storage times. The different antioxidant behavior of an anthocyanin as affected by the pH of the test solution was explained by the ability of the anthocyanin to form the flavylium cation structure or the phenoxide anion. These findings give a criterion for selecting an antioxidant assay in order to screen for the antioxidant activity of a natural extract that may contain anthocyanins. The effect of the test solution pH on the antioxidant activity of the interested compounds should be considered. The results of this study suggested the antioxidative benefit of anthocyanins, which extend the utilization of anthocyanins as antioxidants without limitation due to the pH of the products.

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