Kor. J. Microbiol. Biotechnol.
Vol. 36, No. 1, 55–60(2008)

Isolation of Anthocyanin from Black Rice (Heugjinjubyeo) and Screening of its Antioxidant Activities

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Colored rices are a hulled grains having red or purple pigments in bran. Especially black rice (*Heugjinjubyeo*) is considered to be a healthy food in Asia. Black rice is of great interesting because of the possible biological activity with their anthocyanins. Anthocyanins are water-soluble plant pigments and representatives of flavonoids. The anthocyanins in black rice include cyanidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, pelagonidin 3-O-glucoside and delphinidin 3-O-glucoside. In this study, anthocyanins in a black rice were analyzed quantitatively and qualitatively with HPLC and UV-Vis spectrophotometer. The anthocyanins contained approximately 95% of cyanidin -3-O-glucoside and 5% of peonidin -3-O-glucoside. Antioxidant activities of the anthocyanin extract were investigated by using various in vitro methods. The 100g/ml concentration of the anthocyanin extracted exhibited 88.83% inhibition on the peroxidation of linoleic acid, 55.20% DPPH free radical scavenging activity, 54.96% superoxide anion radical scavenging activity, and 72.67% hydrogen peroxide scavenging activity. And it also showed high ferrous ion reducing capability. These results suggest that the anthocyanin extracted from black rice may be utilized as a possible antioxdiant agent against ROS.

Key words: Anthocyanin, antioxidant, HPLC, ROS(Reactive Oxygen Species)

Introduction

Rice is the principle cereal food in Asia and the staple food of nearly half of the world's population [8]. Colored rice is a hulled grain having red or purple color in addition to light gray on its bran, especially black rice has long been consumed in Korea, Japan and China and is considered to be a healthy food in Korea [8, 10, 13]. It is reported that black rice (*heugjinjubyeo*) contains a lot of anthocyanin pigments [14]. Anthocyanins are belong to water-soluble plant pigments and representatives of flavonoids. They are responsible for the blue, purple and red color of many plant tissues. Depending on the pH, anthocyanins are red to purple or blue [10]. They play a definite role in the attraction of animals for pollination and seed dispersal, and hence they are of considerable value in the co-evolution of these plant-animal interactions [7]. They occur primarily as glycosides of their respective aglycone anthocyanidin-chromophores [7]. The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule [7]. Several researchers have reported that anthocyanins have antioxidant, free radical scavenging activities. [10]. Anthocyanins in berry extract protect DNA integrity and bolster tissue antioxidant levels. And studies have shown that dietary supplementation with berries rich in anthocyanins were effective in reducing oxidative stress [1]. And it has been shown that anthocyanins may reduce the risks of cardiovascular diseases and cancer with anti-inflammatory, antioxidant and chemoprotective properties. *Hibiscus sabdariffa* extract is reported to inhibit low-density lipoprotein oxidation in vitro. Delphinidin could inhibit cell invasion of human fibrosarcoma HT-1080 cell *in vitro*. And both anthocyanins and phenolic compounds inhibit tumor cells proliferation, induced apoptosis in HCT116 human colon carcinoma cells and HL60 human leukemia cells [3].

Aerobic respiratory organisms use of oxygen to produce energy for living, but reactive oxygen species ROSs are also generated during the oxidative metabolisms [4]. ROSs

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may induce cellular damage when ROSs increased and antioxidant defense systems are overwhelmed. This condition is defined as 'oxidative stress' [15]. Damage to cell membranes by lipid peroxidation, damage to DNA, sulfur-containing enzymes and proteins, and carbohydrates are among the major resultant effects [15]. Therefore, ROSs play a crucial role in a wide range of common diseases and age-related degenerative conditions including cardiovascular disease, inflammatory conditions, and neurodegenerative diseases such as Alzheimer's disease, mutations and cancer [2]. So organisms have antioxidant enzymes to protect themselves from oxidative stress such as superoxide dismutase, catalase, and glutathione peroxidase [5]. And there are many natural antioxdiatns. For example, vitamin E, often represented by α-tocopherol, contribute an e^- to the peroxyl radical that is formed during the chain reaction of lipid peroxidation [12].

In present study, anthocyanin extracted from black rice (*Heugjunjubyeo*) by using Amberlites XAD7 column chromatography were analyzed it with HPLC and pH-differential methods, respectively. And we measured antioxidant activities by *in vitro* methods such as DPPH free radical scavenging assay, Superoxide anion free radical scavenging assay, hydrogen peroxide scavenging assay and so on.

Materials and Methods

Isolation of anthocyanin from black rice

Ethanol extraction method: Milled black rice (*heugjinjubyeo*) was obtained from Rural Development Administration of Korea. Milled black rice bran (0.5 g) was soaked in 50 mL of ethanol containing 0.1% Tri fluoro aectic acid for 24 hours. The ethanol extract was pre-filtered with Whatman No.4 filter paper and then evaporated using a vacuum rotary evaporator at 30°C [14].

Amberlite XAD7 column chromatography: For further purification I used column chromatography. The ethanol extract was dissolved in 100 mL of purified water and applied to a column packed with Amberlite XAD7 resin. The column was eluted with 300 mL of 0.3% HCl to remove polar constituents and then eluted sequentially with 10:90(v/v) methanol:water containing 0.3% HCl, 20:80(v/v) methanol:water containing 0.3% HCl, 30:70(v/v) methanol: water containing 0.3% HCl, 40:60 (v/v) methanol:water containing 0.3% HCl, 50:50 (v/v) methanol:water containing 1% TFA, and 60:40(v/v) methanol:water containing 1% TFA. Among the fractions the anthocyanin extract was obtained from 50:50(v/v) methanol:water containing 1% TFA fraction [17]. This isolated anthocyanin extract was used for further study.

HPLC analysis: The anthocyanin extracted was analyzed with HPLC system (Waters, USA) equipped with a data processing computer-Millennium 32.

Quantification of total anthocyanin: The total anthocyanin contents in the black rice was measured by a pH-differential method [9]. Anthocyanin extract of the black rice in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) were measured at 510 and 700 nm, respectively. The content of total anthocyanin was calculated using the following formula:

Anthocyanin contents (mg/liter)
$= (A \times MW \times DF \times 1000)/(e \times 1)$ A
$= (A_{535} - A_{700})pH1.0 - (A_{535} - A_{700})pH4.5$

MW=449.2
e=26,900.

Antioxidant activity of anthocyanin extract

DPPH free radical scavenging activity: The free radical scavenging activity of the anthocyanin extract with column chromatography was measured by DPPH free radical scavenging assay [6]. One ml of 0.1 mM solution of DPPH free radical in ethanol was mixed with 3 mL of aqueous solution of anthocyanin extract (10, 50, 100 μg/mL). Mixed solutions were incubated for 30 min and then absorbance was measured at 517 nm. Lower absorbance indicates higher free radical scavenging activity. The DPPH free radical scavenging activity of pigmented fraction was calculated using the following formula:

DPPH free radical scavenging activity (%)
$= [(A_0 - A_1 / A_0) \times 100]$

A_0 = the absorbance of the control
A_1 = the absorbance in the presence of anthocyanin extract or standards.

Superoxide aninon free radical scavenging activity: Superoxide anion radical scavenging activity was also measured using PMS-NADH system [16]. The superoxide radicals were generated in 3 mL of Tris-HCl buffer (16

mM, pH 8.0) containing 1 mL of NBT (50 M) solution, 1 mL NADH (78 M) solution and the anthocyanin extract in distilled water at 10, 50, and 100 g/mL concentrations. The reaction was started by adding 1ml of PMS solution (10 M) and the reaction mixture was incubated for 5min at 25°C. The absorbance was measured at 560 nm. L-Ascorbic acid was used as a control. Lower absorbance indicates higher superoxide radical scavenging activity. The superoxide anion radical scavenging activity was calculated using the following formula:

Superoxide anion radical scavenging activity (%)
$= [(A_0 - A_1/A_0) \times 100]$,

A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the anthocyanin extract or standards.

Total antioxidant activity with the FTC method: The antioxidant activity of anthocyanin extract was determined using FTC method described by Shanmugsundaram *et al.* [16]. A mixture of 4 mL of 100 g/mL pigmented fraction, 4.1 mL of 2.52% linoleic acid in absolute ethanol, 8 mL of phosphate buffer (0.05 M, pH 7.0) and 3.9 mL of diluted water placed in a test tube with a screw cap was placed in an oven at 40 in the dark. To 0.1 mL of this solution 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate was added. Precisely 3min after the addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm. For every 24 h until the absorbance of the control reached maximum.

Reducing power: The reduction capability of the anthocyanin extract was determined according to the method of Gulcin I *et al.* [6]. The one ml of anthocyanin extract in distilled water (10, 50, and 100 g/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixture was incubated for 20 min at 50°C. 10% trichloroacetic acid (2.5 mL) was added to the mixture, and then centrifuged for 10 min at 1000 g. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Hydrogen peroxide scavenging activity: A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The anthocyanin extract (1, 10, 50, 100 g/mL) in 1.6 mL phosphate buffer (pH 7.4) was added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of H_2O_2 scavenging of anthocyanin extract was calculated using the following formula:

Hydrogen peroxide scavenging activity (%)
$= [(A_0 - A_1)/A_0] \times 100$,

A_0 = the absorbance of the control
A_1 = the absorbance in the presence of the sample of anthocyanin extract [11].

Statistical analysis: All values were represented as means ± S.E.M. Data were analyzed by ANOVA according to General Linear Model procedure. The means were compared by Tukey's Studentized Range (HSD) test to detect significant differences at $P < 0.05$. All statistical procedures were performed with the SAS software package (Release 8.02, 2001).

Results and Discussion

Isolation of anthocyanin from black rice

Anthocyanins were extracted from black rice ethanol extract by Amberlite XAD7 chromatography. The anthocyanin was obtained from 50:50(v/v) methanol:water containing 1% TFA fraction. Total anthocyanin content in the black rice was 1214.85 mg CGE(cyanidin 3-O-glucoside equivalents)/kg of black rice. Also, we analyzed anthocyanin extract with HPLC method using gradient system. The anthocyanin extract HPLC chromatogram has two major peaks. One peak is cyanidin 3-O-glucosde and the other is peonidin 3-O-glucoside.

Antioxidant activity of anthocyanin extract

In the DPPH free radical scavenging assay, anthocyanin extract exhibited 17.65% scavenging activity at 10 μg/mL, 40.39% scavenging activity at 50 μg/mL, and 55.20% scavenging activity at 100 μg/mL, respectively. Fig. 1 shows the result of DPPH free radical scavenging activities of the anthocyanin extract, BHA, BHT, and α-tocopherol. The DPPH free radical scavenging activities of anthocyanin extract, BHA, BHT, and α-tocopherol decreased in the

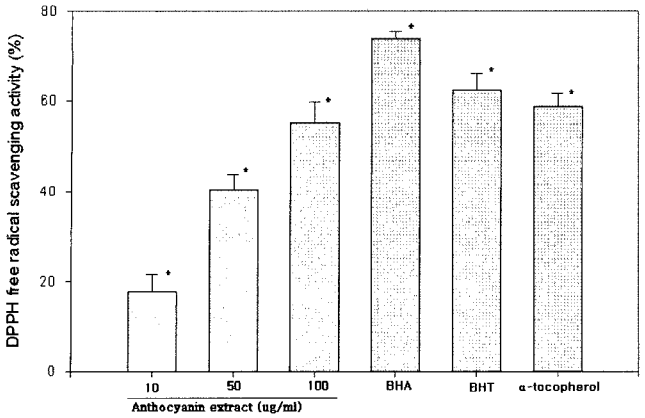

Fig. 1. DPPH free radical scavenging activities of the anthocyanin extract, BHA, BHT, α-tocopehrol. The DPPH free radical scavenging activities of anthocyanin extract, BHA, BHT, and α-tocopherol decreased in the order: BHA (73.80%), BHT (62.43%), α-tocopherol (58.80%), and the anthocyanin extract (55.20%) at 100 μg/mL concentration. These values were very significantly different (P<0.01) from the control.

order: BHA (73.80%), BHT (62.43%), α-tocopherol (58.80%), and the anthocyanin extract (55.20%) at 100 μg/mL concentration. These values were very significantly different (P<0.01) from the control.

Superoxide anion radical scavenging activity was also measured. The anthocyanin extract exhibited 19.78% scavenging activity at 10 μg/mL, 34.61% scavenging activity at 50 μg/mL, and 54.96% scavenging activity at 100 μg/mL, respectively. Fig. 2 shows the result of superoxide anion radical scavenging activities of the anthocyanin extract, BHA, BHT, and α-tocopherol. The anthocyanin extract had higher scavenging activity than α-tocopherol (32.68%) and lower scavenging activity than BHA (69.67%), and BHT (64.61%) at 100 μg/mL concentration. All values were statistically very significant (P<0.01) from the control.

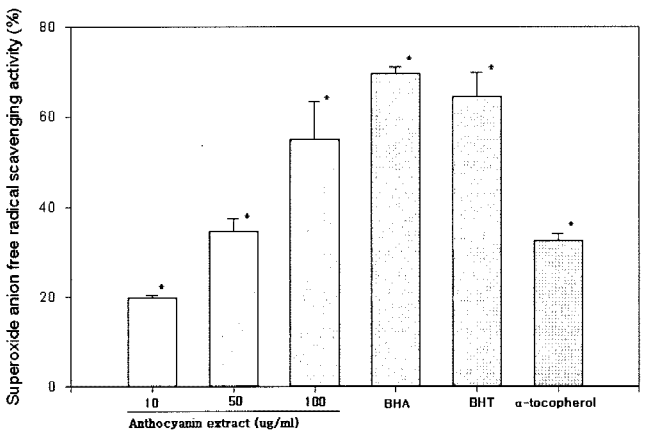

Fig. 2. Superoxide anion free radical scavenging activities of the anthocyanin extract, BHA, BHT, α-tocopherol. The anthocyanin extract had higher scavenging activity than α-tocopherol (32.68%) and lower scavenging activity than BHA (69.67%), and BHT (64.61%) at 100 μg/mL concentration. All values were statistically very significant (P<0.01) from the control.

Fig. 3 shows that anthocyanin extract have high antioxidant activity. It shows more than 90% inhibition on linoleic acid emulsion peroxidation.

Fig. 4 shows the reducing power of the anthocyanin extract, BHA, BHT, and α-tocopherol. The reducing power

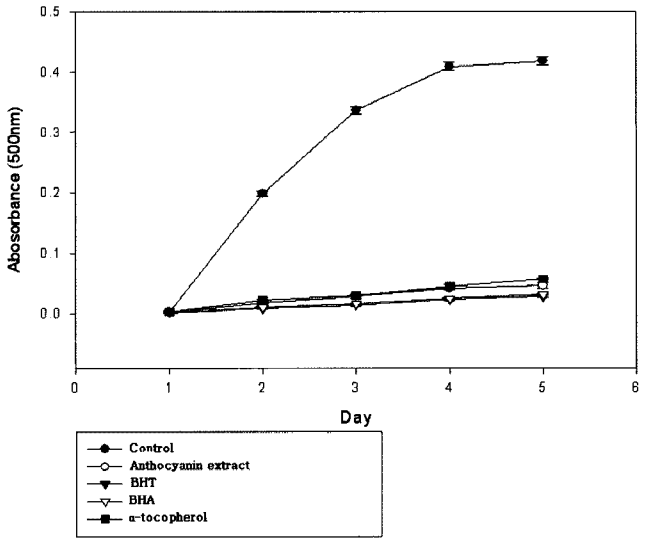

Fig. 3. Total antioxidant activities of the anthocyanin extract, BHA, BHT, α-tocopehrol. It shows more than 90% inhibition on linoleic acid emulsion peroxidation.

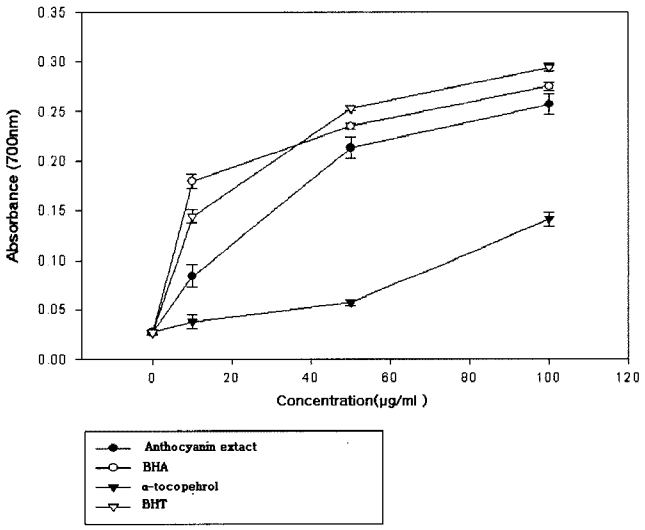

Fig. 4. Reducing power of the anthocyanin extract, BHA, BHT, α-tocopehrol. The reducing power of the anthocyanin extract, BHA, BHT, and α-tocopherol increased with concentration-dependent manner. At 100 μg/mL concentration, the anthocyanin extract shows higher reducing power than α-tocopherol and lower reducing power than BHT and BHA.

of the anthocyanin extract, BHA, BHT, and α-tocopherol increased with concentration-dependent manner. At 100 μg/mL concentration, the anthocyanin extract shows higher reducing power than α-tocopherol and lower reducing power than BHT and BHA.

Hydrogen peroxide scavenging activities of the anthocyanin extract, BHT, BHA, and α-tocopherol was measured at 230 nm. Hydrogen peroxide scavenging activities of the anthocyanin extract were 18.39% at 10 μg/mL, 26.68% at 50 μg/mL, and 72.67% at 100 μg/mL, respectively. Fig. 5 shows that anthocyanin extract had higher hydrogen peroxide scavenging activity than BHA (52.27%), BHT (64.79%), and α-tocopherol (56.99%). All values were statistically different ($P<0.01$) with significance value of the control.

These results suggest that black rice (*Heugjinjubyeo*) contains high amounts of anthocyanins, especially cyanidin 3-O-glucoside which have strong antioxidant activities *in vitro* system.

From the results It can be concluded that *Heugjinjubyeo* has high amount of anthocyanin contents on its bran and anthocyanins can be isolated from the *Heugjinjubyeo* by Amberlites XAD7 chromatography successively.

ROSs plays a crucial role in a wide range of common diseases and age-related degenerative conditions including cardiovascular disease, inflammatory conditions, and neurodegenerative diseases such as Alzheimer's disease, mutations and cancer [8]. So antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components. In this study, the antioxidant activity of the anthocyanin extract was evaluated and it showed very strong antioxidant activity.

Thus, these results suggest that anthocyanin extract from black rice can be used as antioxidant material, food addictives. We suggest that use of anthocyanin extract from black rice may offer an attractive new antioxidant agent against ROS.

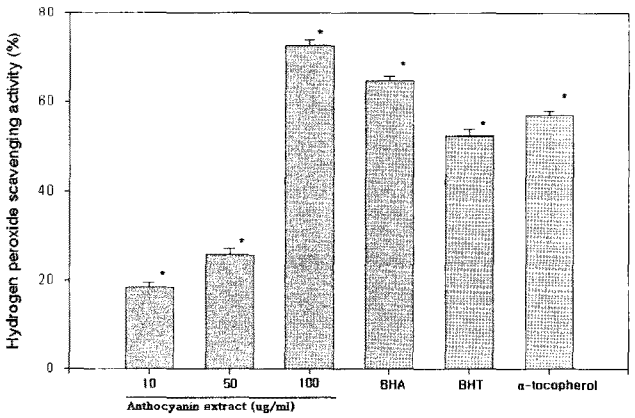

Fig. 5. Hydrogen peroxide scavenging activities of the anthocyanin extract, BHA, BHT, α-tocopehrol. Anthocyanin extract had higher hydrogen peroxide scavenging activity than BHA (52.27%), BHT (64.79%), and α-tocopherol (56.99%). All values were statistically different ($P<0.01$) with significance value of the control

Acknowledgments

This study was supported by the Biogreen 21 from the Korean Rural Development Promotion.

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(Received Oct. 30, 2007/Accepted Dec. 26, 2007)

초록국문

흑미(현진주벼)유래 안토시아닌의 항산화능 탐색

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유색미는 회색 빛 외에 붉은색 혹은 보라색 계통의 색소를 겨 부분에 함유 하고 있다. 특히, 흑미는 예로부터 건강식품으로 여겨져 왔다. 흑미(흑진주벼)는 최근에 anthocyanin 포함하는 생리활성 물질원으로서 관심이 증대되고 있다. Anthocyanin은 수용성의 색소로서 flavonoid 계통의 대표 색소로 알려져 왔다. 흑미에 함유되어있는 Anthocyanin으로는 cyanidin 3-O-glucoside와 peonidin 3-O-glucoside, malvidin 3-O-glucoside, pelagonidin 3-O-glucoside, delphinidin 3-O-glucoside등이 알려져있다. 본 연구에서는, 흑미로부터 amberlite XAD7 chromatography 방법을 사용해서 anthocyanin을 추출 후 HPLC와 UV-Vis spectrophotometer를 이용하여 정량분석 및 정성분석을 실시하였다. 그 결과 흑미의 anthocyanin 추출물 중 95%가 cyanidin 3-O-glucoside, 5%가 peonidin 3-O-glucoside임을 확인하였다. 그리고 *in vitro* 상의 여러 가지 실험방법을 통하여 anthocyanin의 항산화 효과를 측정하였다. 그 결과 100g/ml 농도의 anthocyanin 추출물은 90%이상 linoleic acid emulsion의 과산화를 억제하였고, 55.20%의 DPPH 자유 라디칼 제거효과, 54.96%의 superoxide 자유 라디칼 제거효과, 72.67%의 과산화수소 제거효과를 보였다. 그리고 또한 높은 환원 능력을 확인할 수 있었다. 표준 항 산화 물질로는 BHA, BHT, α-tocopherol을 사용하였다. 이 결과로 보아 anthocyanin추출물은 새로운 항산화 물질로서 이용될 수 있을 것이다.