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Identification and Some Properties of Anthocyanin Isolated from Zuiki, Stalk of *Colocasia esculenta*

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Zuiki, a stalk of taro (*Colocasia esculenta*), is a traditional vegetable in Japan. Raw zuiki is often boiled and vinegared to eat. The surface color of zuiki is reddish. Here, we isolated a red pigment from zuiki and identified it as cyanidin 3-rutinoside using instrumental analyses. The color of zuiki disappeared by boiling, but the zuiki turned red again in an acetic acid solution. It seems that the cyanidin 3-rutinoside that exists on the surface of zuiki elutes in boiling water and then, the pigment that seeps out from the inside of the zuiki is exposed to an acid solution, and its surface turns red again. The radical scavenging activity of purified zuiki anthocyanin was 114 mg equivalent to BHT/g. About half of the anthocyanin in fresh zuiki was washed out by boiling, and the radical scavenging activity of zuiki was definitely reduced.

KEYWORDS: Zuiki (*Colocasia esculenta*); anthocyanin; cyanidin 3-rutinoside; antioxidative activity; boiling

INTRODUCTION

Zuiki, a stalk of taro (*Colocasia esculenta*), is a traditional vegetable in Japan. Raw zuiki is often boiled and vinegared to eat with vegetables, fish, and/or meat. Zuiki is also often dried for storage. Before it is eaten, it is added with water and boiled. Raw zuiki contains 94.5% of water, 0.5% of protein, 4.1% of carbohydrate, and 0.9% of ash, while dried zuiki contains 9.9% of water, 6.6% of protein, 63.5% of carbohydrate, and 18.2% of ash (*1*). The most characteristic feature of zuiki is the color of its surface, which is reddish and turns brilliant red when it is vinegared. This phenomenon suggests that the color of zuiki is derived from anthocyanins.

Anthocyanins are the most important group of water-soluble plant pigments visible to the human eye. They belong to the most widespread class of phenolic compound named flavonoids. Anthocyanins are almost universal colorants of plants and are largely responsible for the brilliant orange, pink, scarlet, red, violet, and blue colors of fruits and flowers. They may also occur in other plant organs such as roots and leaves, accumulating vacuoles (*2*). Anthocyanins are attractive colors of foods as well as antioxidants. Biological activities of anthocyanins are associated with their antioxidant activities, which lead to the prevention of ascorbic acid oxidation, protection against free radicals, inhibitory activity against oxidative enzymes, and reduction of the risk of cancer and heart disease (*3*).

The anthocyanins of taro have been identified as pelargonidin 3-glucoside, cyanidin 3-rhamnoside, and cyanidin 3-glucoside (*4*). However, there is no report on the pigment of zuiki. Here, we describe the isolation and identification of the anthocyanins of zuiki and some properties of the pigment such as color and antioxidative activity from the standpoint of food science.

MATERIALS AND METHODS

Materials. Fresh zuiki, cultivated in Saitama and Chiba prefectures in Japan, was purchased from local markets in Tokyo and Chiba prefectures. These zuiki samples were stored at 4 °C within 7 days or lyophilized.

Extraction and Purification of Pigment. Lyophilized zuiki (100 g, about 1860 g of fresh zuiki) was added with 2 L of 80% ethanol and homogenized, soaked overnight, and filtered by a filter paper (no. 2, Toyo Roshi, Tokyo, Japan). This extraction procedure was repeated again. The combined filtrates were diluted four-fold with water and then applied to a Diaion HP-20 column (4 cm i.d. × 35 cm, Mitsubishi Chemical, Tokyo). After the column was washed with 20% ethanol, the absorbed pigment was eluted by 80% ethanol. The eluate was concentrated in vacuo, washed three times with ether, and lyophilized. Then, this crude pigment (4.03 g) was applied to a cellulose column (4 cm i.d. × 39 cm, Merck Cellulose Microcrystalline, Merck, Darmstadt, Germany), which was eluted by *n*-propanol:acetic acid:water (12:1:1, v/v/v). The red eluate was concentrated and lyophilized (1.61 g). The separated pigment was further purified by high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: column, Mightysil RP-18 GP (20 mm i.d. × 250 mm, Kanto Chemical, Tokyo); pump, L-6000 (Hitachi, Tokyo); detector, L-4200 (Hitachi); detection, 535 nm; flow rate, 9.99 mL/min; eluent, methanol−10% formic acid (20:80, v/v). A peak with a retention time of 18 min

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was colleted, concentrated in vacuo, and lyophilized. A reddish powder was obtained (96.2 mg).

Acid Hydrolysis of Anthocyanin. The zuiki anthocyanin (1 mg) was dissolved in 4 N HCl (1 mL) and methanol (1 mL) in a test tube with a screw cap, which was heated at 100 °C for 40 min. The resultant solution was cooled, and the formed aglycone was extracted with amyl alcohol (2 mL) three times (*5*). The amyl alcohol portion of hydrolyzate was concentrated in vacuo, dissolved into methanol, and applied to HPLC and liquid chromatography/mass spectrometry (LC/MS) to analyze the aglycone. The remaining aqueous phase was concentrated in vacuo and applied to silica gel thin-layer chromatography (TLC) (Silica gel 60 F_{254}, Merck; *n*-butanol:acetic acid:water = 4:1:5 upper phase) with authentic sugars such as glucose, galactose, rhamnose, xylose, and arabinose. Sugars were detected by the anisaldehyde reagent consisting of 0.25 mL of anisaldehyde, 25 mL of acetic acid, and 0.5 mL of sulfuric acid (*5*).

The HPLC conditions for aglycone analysis were as follows: column, Mightysil RP-18 GP (6.0 mm i.d. × 250 mm, Kanto Chemical); pump, L-6320 Intelligent Pump (Hitachi); detector, L-4500 diode array detector (Hitachi); detection, 535 nm; integrator, D-6100 (Hitachi); flow rate, 1.00 mL/min; and eluent, gradient solvent system consisting of solvent A (10% formic acid) and solvent B (methanol). The elution profile was as follows: 0−33 min, 5−60% B; 34−42 min, 60−100% B; and 43−50 min, 100% B. Cyanidin and the aglycone of the zuiki pigment were eluted at 31 min. The LC/MS conditions were as follows: LCMS-2010A (Shimadzu, Kyoto, Japan); column, Shim-pack VP-ODS (2.0 mm i.d. × 250 mm, Shimadzu); column oven, 40 °C; pump, LC-10ADvp (Shimadzu); detector, SPD-M10Avp (Shimadzu); flow rate, 0.12 mL/min; and eluent, gradient solvent system consisting of solvent A (5% acetic acid:methanol = 55:45 v/v) and solvent B (methanol). The elution profile was as follows: 0−22 min, 0% B; 22−30 min, 0−100% B; and 30−40 min, 100% B. Pelargonidin chloride, cyanidin chloride, peonidin chloride, and delphinidin chloride (Extrasynthese, Genay, France) were used as standard samples.

Instrumental Analysis of the Anthocyanin. The isolated zuiki anthocyanin was analyzed by a spectrophotometer (U-3310, Hitachi), NMR (AVANCE800, Bruker Biospin, Karlsruhe, Germany), and electrospray ionization mass spectrometry (FT-ICR, ApexII 70e, Bruker Daltonics, Billerica, MA).

Effect of Heat and Acid Treatment on the Color of Zuiki and Anthocyanin Content. Fresh zuiki was cut into about 1.5 cm i.d. × 2 cm (about 1.3 g) and heated for 2 or 10 min in 20 mL of boiling water. After the boiled zuiki was drained, it was soaked in a 5% acetic acid solution or distilled water for 1−24 h. The color of the surface of the zuiki was estimated by the L^*, a^*, and b^* values using a colorimeter (NF333, Nippon Denshoku, Tokyo), and the pH of the surface of the zuiki was measured using a microelectrode (6261-10C, Horiba, Kyoto).

Zuiki samples (about 1.5 g), before and after boiling, were homogenized in 10 mL of 80% ethanol and extracted twice with 80% ethanol. The combined extracts were concentrated in vacuo and dried up. This extract (10 mg/mL) was applied to analytical HPLC, and the content of cyanidin 3-rutinoside was determined. The HPLC conditions were as follows: column, Mightysil RP-18 GP (6.0 mm i.d. × 250 mm, Kanto Chemical); pump, L-6320 (Hitachi); detector, L-4500 diode array detector (Hitachi); detection, 535 nm; flow rate, 1.00 mL/min; and eluent, gradient solvent system consisting of solvent A (10% formic acid) and solvent B (methanol). The elution profile was as follows: 0−33 min, 5−60% B; 34−42 min, 60−100% B; and 43−50 min, 100% B. Cyanidin 3-rutinoside was eluted at 18−19 min. The total amount of anthocyanins of zuiki was estimated from the absorbance of the ethanol extract. After 2 mL of 1% trifluoroacetic acid was added to 0.5 mL of the extract, its absorbance at 535 nm was measured by a spectrophotometer.

Vitamin C Content. Raw or boiled zuiki (5 g) was homogenized in a solution containing 1% tin chloride and 5% metaphosphoric acid, and the amounts of ascorbic acid and dehydroascorbic acid were measured by the 2,4-dinitrohydrazine method (*6*).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity. The radical scavenging activity of anthocyanin of zuiki was investigated by DPPH method (*7*, *8*). The purified anthocyanin was dissolved in 0.1 M acetate buffer (pH 5.5) at concentrations of 50 and 100 μg/mL. The anthocyanin solution (2 mL), ethanol (2 mL), and 0.5 mM DPPH−ethanol (1 mL) were mixed in a test tube. A blank was made up with ethanol added instead of the DPPH/ethanol solution, and a control was made up with the acetate buffer added in place of the sample solution. The radical scavenging activity (%) was calculated by the following formula:

$$\{A - (B - C)\}/D \times 100$$

where A = absorbance at 517 nm (A_{517}) of the control, which was measured immediately after preparation; $B = A_{517}$ of the sample solution, which was measured 30 min after preparation; $C = A_{517}$ of the blank, which was measured 30 min after preparation; and $D = A_{517}$ of the control, which was measured 30 min after preparation. The relationship between concentration of dibutyl hydroxytoluene (BHT) and DPPH radical scavenging activity was measured and used as the standard curve.

Statistical Analysis. A statistical analysis of variance for each sample was done by Statcel2 (OMS, Tokyo, Japan) on Microsoft Office Exel 2003 (Microsoft). Differences at $p < 0.05$ were considered significant.

Table 1. NMR Data of Zuiki Anthocyanin[a]

	δ_H	J (Hz)	δ_C
	cyanidin		
2			162.1
3			144.5
4	8.80 (s)		134.5
4a			112.2
5			157.0
6	6.70 (s)		102.5
7			168.4
8	6.88 (s)		94.3
8a			156.0
1′			119.9
2′	7.98 (d)	2.2	117.7
3′			146.3
4′			154.5
5′	7.02 (d)	8.7	117.0
6′	8.21 (dd)	8.7, 2.2	127.2
	glucose		
1″	5.35 (d)	7.8	102.0
2″	3.48 (m)		73.2
3″	3.37 (m)		76.2
4″	3.21 (m)		69.9
5″	3.66 m)		76.4
6″	3.87/3.41 (d)	10.7	66.5
	rhamnose		
1‴	4.50 (s)		100.9
2‴	3.57 (m)		70.5
3‴	3.41 (m)		70.9
4‴	3.12 (m)		72.2
5‴	3.36 (m)		68.7
6‴	1.03 (s)		18.0

[a] Sample was dissolved in a mixture of DMSO-d_6 and TFA-*d* (9:1, v/v).

RESULTS AND DISCUSSION

Isolation and Identification of Zuiki Anthocyanin. A red pigment was isolated from zuiki. The yield of the purified anthocyanin from 1860 g of fresh zuiki was 96.2 mg. The instrumental data of this pigment were as follows: UVλ_{max} (0.1% HCl−MeOH) nm (ϵ): 525 (1560). LC/MS *m*/*z*: 596 $(M + 1)^+$. ^{1}H NMR δ_H (DMSO-d_6:TFA-*d* = 9:1) and ^{13}C NMR δ_C (DMSO-d_6:TFA-*d* = 9:1) are shown in **Table 1**.

The UV spectrum of purified pigment suggests that the zuiki pigment is an anthocyanin. The aglycone of the zuiki anthocyanin obtained by hydrolyzate was determined as cyanidin by the retention time and UV−visible spectrum of diode array detection on HPLC and molecular-related ions on LC-MS [*m*/*z*; 287 (M^+), 285 (M − 2H)]. The percent of the absorbance at

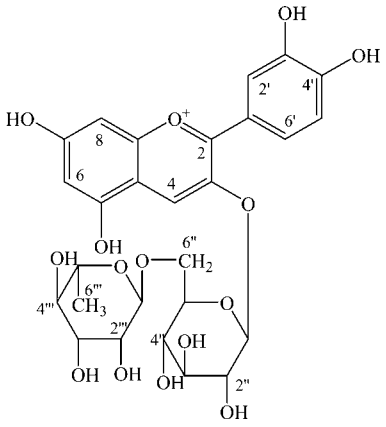

Figure 1. Structure of zuiki anthocyanin, cyanidin 3-rutinoside.

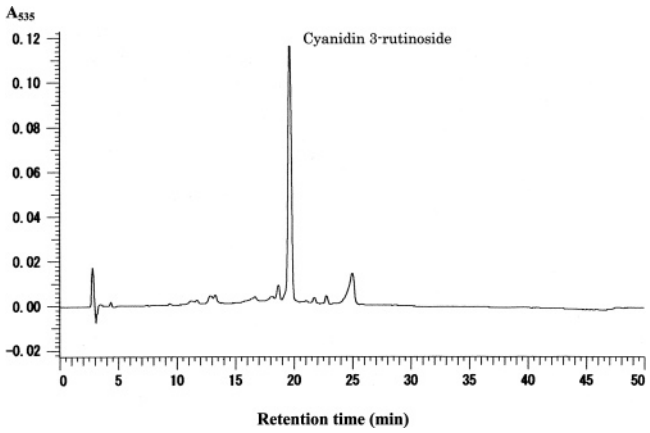

Figure 2. Typical pattern of HPLC of 80% ethanol extract of fresh zuiki.

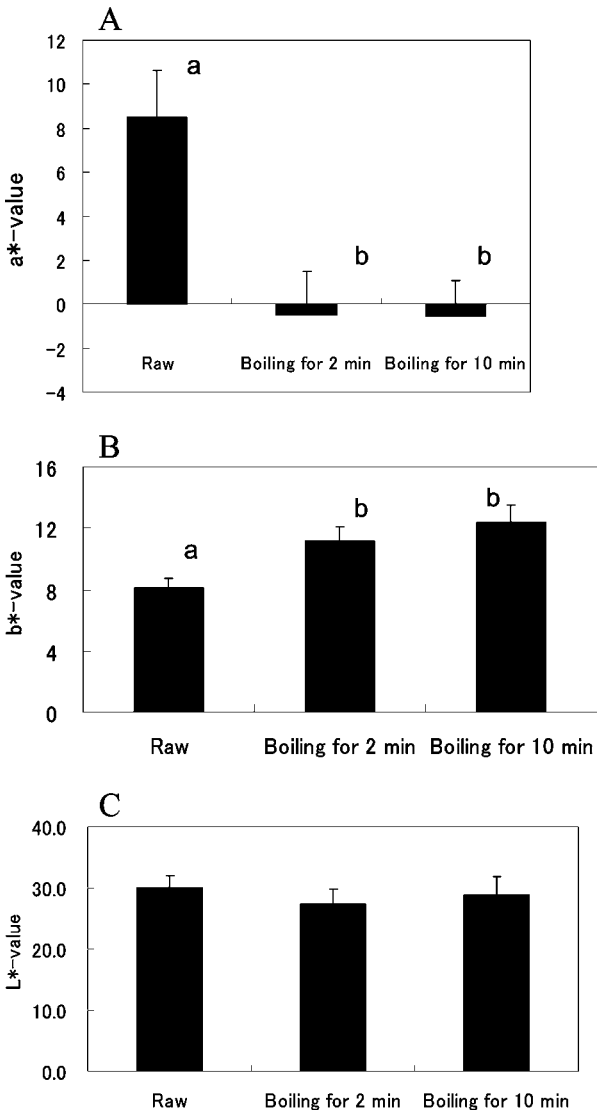

Figure 3. Effect of boiling on the surface color of zuiki. (**A**) *a** value, (**B**) *b** value, and (**C**) *L** value. Different letters (a and b) show a significant difference ($p < 0.05$, $n = 3$).

440 nm to that of the absorption maximum ($E_{440}/E_{max} \times 100$) of the zuiki anthocyanin and cyanidin 3-glucoside was 23, while those of cyanidin and cyanidin 3,5-diglucosides were 46 and 12, respectively, which suggests that the position of glucosylation is C-3 (*5*). Rhamnose and glucose were detected from the hydrolyzate on TLC. These data suggest that the zuiki pigment is cyanidin 3-rutinoside (**Figure 1**).

To ascertain this, the sample was applied to NMR analyses. Six proton signals at δ_H 6.70−8.80 ppm, 12 proton signals at δ_H 3.12−5.35 ppm, and a methyl proton signal at δ_H 1.03 ppm detected in ^{1}H NMR, respectively, corresponded to protons of cyanidin and those of sugars. ^{1}H−^{1}H correslation spectroscopy showed the two correlations of signals at δ_H 5.35, 3.48, 3.37, 3.21, 3.66, and 3.87/3.41 ppm and signals at δ_H 4.50, 3.57, 3.41, 3.12, 3.36, and 1.03, which corresponded to glucose and rhamnose, respectively. In the ^{13}C NMR spectrum, 17 signals were detected at δ_C 94.3−168.4 ppm, 15 signals of which corresponded to carbons of cyanidin. The connection of each carbon and proton was assigned by ^{13}C−^{1}H heteronuclear single quantum correlation analysis. Two signals at δ_C 102.0 and 100.9 ppm, respectively, corresponded to C1 of glucose and C1 of rhamnose. ^{13}C−^{1}H heteronuclear mulitple bond correlation analysis then showed that the proton at δ_H 3.41 ppm (H6 of glucose) and the carbon at δ_C 100.9 ppm (C1 of rhamnose) and the proton at δ_H 5.35 ppm (H1 of glucose) and the carbon at δ_C 144.5 ppm (C3 of cyanidin) were, respectively, correlated. These data show that position 1 of rhamnose and position 6 of glucose are connected and that position 1 of glucose and position 3 of cyanidin are connected. The MS analysis shows that the molecular weight of this pigment is 595. From these data, zuiki anthocyanin was identified as cyanidin 3-rutinoside (**Figure 1**).

The content of cyanidin 3-rutinoside in zuiki was estimated by HPLC analysis. As shown in **Figure 2**, cyanidin 3-rutinoside was the major anthocyanin and occupied more than 70% of total anthocyanin in zuiki. Fresh zuiki contained about 47 mg/100 g of cyanidin 3-rutinoside. Cyanidin 3-rutinoside has been detected as a minor anthocyanin in several berries and fruits such as strawberry (*9*), blackberry (*10*), blueberry (*11*), boysenberry (*12*), berry of *Rhamnus alaternus* (*13*), berry of *Smilax aspera* (*14*), olive fruits, (*15*) sweet cherry (*16*), blue honeysuckle (*17*), apple skin (*18*), banana bract (*19*), etc. It has also been detected in litchi (*Litchi chinensis*; *20*, *21*), black currant (*22*), and bay berry (*23*) as the major anthocyanin. Lich peel contained about 30 mg/100 g (*20*) of cyanidin 3-rutinoside. Here, cyanidin 3-rutinoside was detected as the major pigment in zuiki. Zuiki is the stalk of taro. There is no report that stalks contain cyanidin 3-rutinoside.

Effect of Heat and Acid Treatment on Zuiki Pigment. Zuiki is usually boiled and vinegared to eat. Therefore, the effect of boiling and acid treatment on zuiki color was examined. The reddish color (*a** value) of the surface of zuiki disappeared by boiling (**Figure 3**), while *L** and *b** values were unchanged and slightly changed, respectively. The pH of the surface of zuiki increased up to pH 6.4 by boiling (**Figure 4**), which suggests that the reddish color of zuiki disappears because

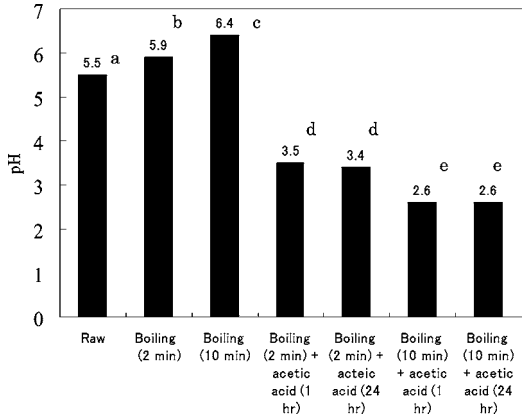

Figure 4. Change in pH of the zuiki surface by boiling and acetic acid treatment. Zuiki was boiled for 2 or 10 min and then immersed in 5% acetic acid solution for 1 or 24 h. Different letters (a–e) show a significant difference ($p < 0.05$, $n = 3$).

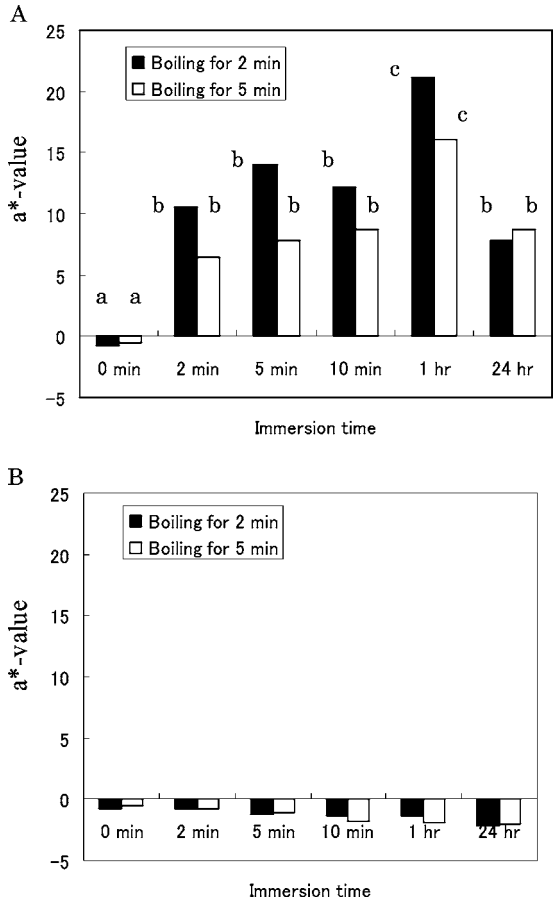

Figure 5. Changes in surface color of boiled zuiki by immersion in 5% acetic acid solution (**A**) and distilled water (**B**). Different letters (a–c) at the same boiling time show a significant difference ($p < 0.05$, $n = 3$).

anthocyanins existing on the surface of zuiki are washed out and the surface is exposed to neutral pH. When boiled zuiki was soaked in an acetic acid solution, the surface of the boiled zuiki turned red again (**Figure 5A**), but it did not turn red when soaked in water (**Figure 5B**). When a peel from boiled zuiki was soaked in an acetic acid solution, it did not turn red (**Figure 6**). Cyanidin is known to turn red in an acidic solution. Therefore, these data suggest that cyanidin 3-rutinoside existing on the surface of zuiki is eluted in boiling water; then, cyanidin 3-rutinoside that seeped out from the inside of zuiki is exposed to an acid solution, and the surface of zuiki turns red.

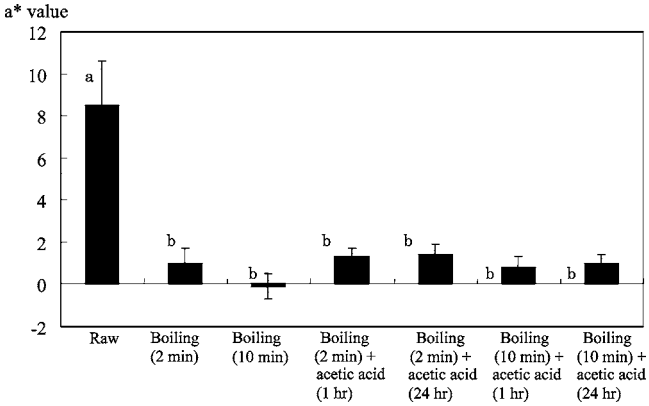

Figure 6. Changes in color of peel of zuiki by boiling and being vinegared in 5% acetic acid solution. Different letters (a and b) show a significant difference ($p < 0.05$, $n = 3$).

Table 2. Changes in Total Anthocyanins by Cooking and Being Vinegared ($n = 3$)[a]

treatment	anthocyanins in zuiki (%)	anthocyanins in boiling water (%)	anthocyanins in vinegar (%)	recovery (%)
control (fresh zuiki)	100 a			100
boiling for 2 min	61.4 b	41.7		103.1
boiling for 10 min	41.3 b	50.1		91.4
1 h in vinegar after boiling for 2 min	35.9 b,c	41.7	18.5	96.1
24 h in vinegar after boiling for 2 min	18.1 b,c	41.7	12.3	72.1
1 h in vinegar after boiling for 10 min	15.5 b,c	50.1	20.1	85.7
24 h in vinegar after boiling for 10 min	8.4 c	50.1	12.6	71.1

[a] The amount of anthocyanins was estimated as the absorbance at 535 nm in the presence of trifluoroacetic acid. A solution of 5% acetic acid was used as a vinegar. Different letters (a–c) in the same column show a significant difference ($p < 0.05$).

The effects of acidification and boiling on zuiki color and anthocyanins were further examined. The reddish color of zuiki increased after it was soaked in an acetic acid solution for 1 h, but it decreased after 24 h (**Figure 5A**). The zuiki pigment might be partly decomposed in an acetic acid solution. It is reported that cyanidin 3-rutinoside is stable at pH 0.6−3.3, while it is partly decomposed at pH 3.8 (*24*). The total amounts of anthocyanins of boiled zuiki and their boiling water for 2 or 10 min were 103 or 91.4%, respectively, which were almost the same levels as fresh zuiki (**Table 2**), while the total amount of anthocyanins of vinegared zuiki for 24 h after 10 min of boiling decreased to about 70%. It seems that zuiki anthocyanin is partly degraded by an acetic acid solution. Zuiki boiled for 2 or 10 min contained only 60 or 40% of anthocyanins of fresh zuiki, respectively, which shows that about a half of anthocyanins of zuiki is washed out to boiling water.

In summary, anthocyanins in zuiki decreased to about half after boiling by leaching out in boiling water. The reddish color of the zuiki surface disappeared due to the neutral pH of boiling water penetrated in the tissue. The reddish color of zuiki was recovered by acidification. This can be explained by seeping out anthocyanins from inner tissues to the surface. Although anthocyanins in zuiki decreased by about half after boiling, the

Table 3. Changes in Contents of Cyanidin 3-Rutinoside and Vitamin C and Radical Scavenging Activity in Zuiki by Cooking and Being Vinegared ($n = 4$)[a]

treatment	cyanidin 3-rutinoside (%)	vitamin C (%)	radical scavenging activity (%)
control (fresh zuiki)	100 a	100 a	100 a
boiling for 2 min	65.3 b	51.5 b	68.1 b
boiling for 10 min	45.2 b,c	28.8 b	50.5 b,c
1 h in vinegar after boiling for 2 min	38.8 c,d	26.4 b,c	39.3 c,d
24 h in vinegar after boiling for 2 min	16.0 d,e	12.3 c	20.1 d,e
1 h in vinegar after boiling for 10 min	22.4 c,d,e	9.1 c	33.4 c,d,e
24 h in vinegar after boiling for 10 min	9.3 d,e	7.0 c	13.6 e

[a] Contents of cyanidin 3-rutinoside and vitamin C in fresh zuiki were 47 and 1.3 mg/100 g, respectively, and the radical scavenging activity of fresh zuiki was 32.3 mgBHT/g. A solution of 5% acetic acid was used as vinegar. Different letters (a–e) in the same column show a significant difference ($p < 0.05$).

total anthocyanins including ones in boiling water did not change. This proves the stability of the pigment in boiling water. However, the total anthocyanins after soaking of 10 min-boiled zuiki in acetic acid solution for 24 h decreased to about 70%, suggesting degradation of the pigment in the acid solution.

Contents of Cyanidin 3-Rutinoside and Vitamin C and Radical Scavenging Activity of Zuiki after Boiling and Being Vinegared. Cyanidin 3-rutinoside is known to show various biological activities such as antioxidant activities (*25*−*27*), α-glucosidase inhibition (*28*), inhibition of cancer migration and invasion (*29*), etc. Here, we estimated the radical scavenging activity of zuiki. Radical scavenging activities of crude and purified anthocyanin of zuiki were 32 and 114 mg equiv BHT/g, respectively. The activity of the pigment of purple-black rice is about 700 mg equiv BHT/g (*30*). The activity of the zuiki pigment was weaker than that of purple-black rice.

Next, the contents of cyanidin 3-rutinocide and vitamin C and the radical scavenging activities of boiled and vinegared zuiki were investigated (**Table 3**). The radical scavenging activity was definitely decreased by boiling. Zuiki contained about 1.3 mg/100 g of vitamin C. About 52 or 29% of vitamin C still remained after 2 or 10 min of boiling, respectively, while about 26 or 7% of vitamin C only remained by 1 h of soaking in 5% acetic acid after 2 min of boiling and by 24 h of soaking in 5% acetic acid after 10 min of boiling, respectively. Cyanidin 3-rutinoside was also definitely decreased by boiling. About 65 or 45% of cyanidin 3-rutinoside remained after 2 or 10 min of boiling, and only 9% of the pigment remained by being vinegared for 24 h after 10 min of boiling. It seems that the radical scavenging activity of zuiki was decreased by decomposition and/or elution of ascorbic acid and anthocyanins during boiling and being vinegared.

In conclusion, the anthocyanin of zuiki was isolated and identified as cyanidin 3-rutinoside. Fresh zuiki contained about 0.47 mg/g of cyanidin 3-rutinoside. The reddish color of zuiki disappeared by boiling, but the surface turned red again after being soaked in an acetic acid solution. It seems that cyanidin 3-rutinoside existing on the surface of zuiki is eluted in boiling water and that cyanidin 3-rutinoside that seeped out from the inner part is exposed to acid solution, and the surface turns red. The radical scavenging activity of purified zuiki anthocyanin was 114 mg equiv to BHT/g. About half of the pigment was eluted from zuiki by boiling.

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