Antioxidative Pigments Isolated from the Seeds of *Phaseolus vulgaris* L.

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Antioxidative activity of pea bean (*Phaseolus vulgaris* L.) extract was examined. A 0.5% TFA–80% ethanol extract prepared from red and black bean seed coat exhibited strong antioxidative activity. To determine the antioxidative role of the pigments in the seed coats, the pigments, RP0 (cyanidin 3-*O*-β-D-glucoside) and RP1 (pelargonidin 3-*O*-β-D-glucoside) from red bean and BP1 (delphinidin 3-*O*-β-D-glucoside) from black bean, were isolated. RP0 showed strong antioxidative activity in the linoleic acid system at neutral condition (pH 7.0), while RP1 and BP1 exhibited no antioxidative activity at pH 7.0. In acidic conditions (pH 3.0 and 5.0), RP1 and BP1 showed strong antioxidative activity. The peak of RP0 at pH 7.0 in the linoleic acid system had disappeared in 10 days according to HPLC analysis. These results suggest that the antioxidative mechanism of RP0 may be different from that of RP1 and BP1.

Lipid peroxidation is known as one of the major factors in deterioration during the storage and processing of food. In addition, it is thought that lipid peroxidation is strongly associated with aging and carcinogenesis (Yagi, 1987; Cutlar, 1984). The addition of antioxidants has become popular as a means of increasing the shelf life of food products and improving the stability of lipids and lipid-containing foods by preventing loss of sensory and nutritional quality. In living systems, dietary antioxidants such as α-tocopherol and ascorbic acid may be effective in protection from oxidative damage.

Effective synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used in food industries; however, these synthetic antioxidants are suspected to be carcinogenic (Branen, 1975; Ito, 1983). Therefore, the importance of natural antioxidants has increased greatly.

Pea bean (*Phaseolus vulgaris* L.) is cultivated throughout the world for its pods and seeds and is consumed in both Western and Asian dishes. However, there are few reports about the antioxidative activity of pea bean seed coat extract. The effects of navy bean (*P. vulgaris* L.) crude hull extracts on the oxidative stability of edible oil were reported by Onyeneho and Hettiarachchy (1991); however, detailed chemical studies of the bean antioxidants are unknown. Therefore, we decided to examine the activity; screening for antioxidative activity of bean seeds was investigated, and antioxidative activity of some kinds of pea bean seeds was discovered (Tsuda et al., 1993a,b).

In general, seed coat may play an important role in chemical protection from oxidative damage by possessing endogenous antioxidants such as phenolic compounds (Osawa et al., 1985; Ramarathnam et al., 1989). This background prompted us to investigate antioxidants in the seed coat of bean seeds chemically.

There are many varieties of seeds in *P. vulgaris* L., and the size, shape, and color in each variety generally differ. Color variation—white, red, black—is especially great, but there has been no attention to pigments from the viewpoint of antioxidative defense systems. To determine if these components play an important role in antioxidative activity for protection against oxidative damage in bean seeds, the pigments from *P. vulgaris* L. were isolated and identified their antioxidative activity in model systems was examined.

MATERIALS AND METHODS

Three varieties of bean seeds (*P. vulgaris* L.) with white, red, and black seed coats were selected for the study. White beans (*P. vulgaris* L. cv. Ohtebo) was obtained from Hirukawa Co. Ltd., Japan. Red beans (*P. vulgaris* L. cv. Honkintoki) and black beans (*P. vulgaris* L. cv. Yamashirokurosando) were purchased from Aisan Seedlings Co. Ltd., Japan. These seeds were cultivated in Hokkaido in 1991, cleaned, and stored at 4 °C until used; all of the samples are commercial varieties consumed in Japan. Linoleic acid was purchased from Tokyo Kasei Kogyo Co., Tokyo. α-Tocopherol and BHA were obtained from Wako Pure Chemical Industries, Ltd. An authentic sample of pelargonidin 3-*O*-β-D-glucoside was purchased from Extrasynthese, France.

Extraction of Antioxidants from Bean Seeds. The three kinds of bean seeds were soaked in 80% ethanol for 30 min at room temperature. The seed coats and germs were then removed manually. The separated seed coats and germs were ground and extracted four times with 0.5% trifluoroacetic acid (TFA)–80% ethanol and then filtered. The filtrates were concentrated to dryness *in vacuo* to obtain crude seed coat extracts and germ extracts.

Antioxidative Assay for Crude Extracts. Antioxidative activity was carried out by using the linoleic acid system (Osawa and Namiki, 1981). Each sample (200 μg) was added to a solution mixture of linoleic acid (0.13 mL), 99.0% distilled ethanol (10 mL), and 50 mM phosphate buffer (pH 7.0, 10 mL); the total volume was adjusted to 25 mL with distilled water. The solution was incubated at 40 °C, and the degree of oxidation was measured according to the thiocyanate method (Mitsuda et al., 1966) by reading the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate and by the thiobarbituric acid (TBA) method (Ottolenghi, 1959). α-Tocopherol (Toc) and BHA were used as standard samples.

Purification of Pigments from Red Bean. The ground seed coat (14.7 g) prepared from red bean seeds (210 g) was extracted with 300 mL of 0.5% TFA–80% ethanol four times to obtain crude seed coat extract (3.3 g). Preparative high-performance liquid chromatography (HPLC) was carried out by using a Develosil ODS-10 column (Nomura Chemical Co. Ltd., Japan, 20 mm × 250 mm) with a spectrophotometric detector (TOSOH UV-8000) with vis (515 nm) or UV (280 nm) and 0.1% TFA–20% acetonitrile as solvent at a flow rate of 5.0 mL/min. Red bean pigment 0 (RP0, 12.1 mg) and red bean pigment 1 (RP1, 102.2 mg) were obtained.

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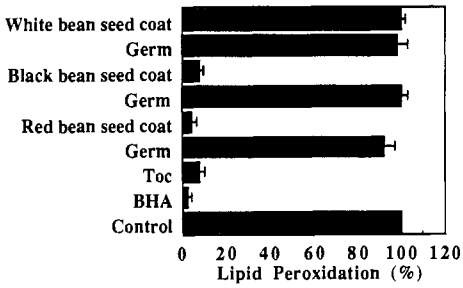


Figure 1. Antioxidative activity of crude extracts prepared from white, red, and black beans, butyl hydroxyanisole (BHA), and α-tocopherol (Toc) (200 μg) as measured by the thiocyanate method. Reported values are the mean ± SD ($n = 4$). A control containing no added samples or standards on its values represents 100% lipid peroxidation.

Purification of Pigment from Black Bean. The ground seed coat (6.1 g) prepared from black bean seeds (68 g) was extracted with 100 mL of 0.5% TFA–80% ethanol four times, and then crude seed coat extract (1.0 g) was obtained. Purification of the pigment was conducted with preparative HPLC using a Develosil ODS-10 column and a detector at vis (535 nm) or UV (280 nm). The solvent system was 0.1% TFA–15% acetonitrile at a flow rate of 5.0 mL/min. Black bean pigment 1 (BP1, 31.1 mg) was obtained.

Instrumental Analysis of Pigments. UV–vis absorption spectra were recorded on a spectrophotometer with a JASCO U best-50 in 0.01% HCl–methanol. IR spectra were recorded on a JASCO FT/IR-8000 with KBr. The fast atom bombardment mass spectra (FAB-MS) were recorded on a JEOL JMS-DX-705L with 1 N HCl–glycerol as the mounting matrix. ^{1}H NMR and ^{13}C NMR spectra were obtained by a JEOL JNM-EX-270 NMR instrument (270 MHz for ^{1}H and 67.5 MHz for ^{13}C) in 10% *d*-CF_3COOD–*d*-CD_3OD containing tetramethylsilane (TMS) as an internal standard for all samples.

Antioxidative Activity of Isolated Pigments in Neutral Condition. Antioxidative activity of the isolated pigments was determined by the thiocyanate method with the linoleic acid system described above. The concentration of pigments was 100 μM in each case. Toc (100 μM) was used as positive control.

Antioxidative Activity of Isolated Pigments in Acidic Conditions. Antioxidative activity of isolated pigments in acidic conditions (pH 3.0 and 5.0) was determined by the thiocyanate method with the linoleic acid system reported by Igarashi et al. (1989). The sample solution (0.1 mL) was added to a mixture of linoleic acid–99% distilled ethanol–50 mM citrate buffer–distilled water; pH 3.0 and pH 5.0 solution mixtures were obtained. pH values were unaffected by the presence or absence of isolated pigments. During incubation at 70 °C, the degree of oxidation was measured by using the thiocyanate method. The concentrations of the pigments and Toc were 100 μM in each case.

Comparison of Antioxidative Activity and Stability of the Pigments. To compare the antioxidative activity and the stability of the pigment, HPLC analysis was carried out. In the linoleic acid system at pH 7.0 containing RP0 (100 μM) described above, the reaction mixture (40 μL) was analyzed by HPLC and the pigment peak area was observed and compared at intervals during incubation. HPLC was carried out by using a Develosil ODS-10 column (4.6 mm × 250 mm) with a UV detector (λ = 280 nm). First, the samples were eluted with an isocratic condition of 0.1% TFA in water for 10 min; then a linear gradient ranging from 0.1% TFA in acetonitrile over 30 min was done, and the samples were eluted with isocratic conditions of 0.1% TFA in acetonitrile for 10 min. The flow rate was 1.0 mL/min.

Statistics. Statistical analysis was performed by using Student's *t*-test.

RESULTS AND DISCUSSION

Antioxidative Activity of Crude Extracts. Antioxidative activities of crude extracts from three differently colored bean seeds as measured by the thiocyanate method are shown in Figure 1. There was no antioxidative activity in the extract prepared from white bean seed coat. On the other hand, seed coat extracts from red beans and black beans exhibited strong antioxidative activity. The extent of activity of red bean and black bean seed coat extract was the same as that of 200 μg of Toc (not significant). There was no antioxidative activity in any of the extracts prepared from germs. The results measured by the TBA method showed the same tendency (data not shown).

From these results, we see that antioxidative substances are present in seed coat and that colored seeds show strong activity.

Characterization of Pigments. UV–vis absorption λ_{max}, IR spectrum, FAB-MS, and ^{1}H NMR of RP0, RP1, and BP1 are shown in Table 1. ^{13}C NMR spectral data of these isolated pigments are shown in Table 2.

Considering these spectral data and the two-dimensional NMR spectra, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, and HSQC (data not shown), RP0 could likely be identified as cyanidin 3-*O*-β-D-glucoside, since the spectral properties obtained from RP0 are also consistent with those of literature data (Hoffmann and Hölzl, 1988). Therefore, RP0 was identified as cyanidin 3-*O*-β-D-glucoside.

RP1 could likely be identified as pelargonidin 3-*O*-β-D-glucoside from the instrumental data as shown in Tables 1 and 2 and the two-dimensional NMR spectra, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, and HSQC (data not shown). To confirm this, the ^{1}H NMR, ^{13}C NMR, UV–vis, and IR spectra of authentic pelargonidin 3-*O*-β-D-glucoside were measured and compared with the chemical shifts of RP1. These authentic spectra were consistent with those of RP1. Therefore, RP1 was identified as pelargonidin 3-*O*-β-D-glucoside.

BP1 could likely be identified as delphinidin 3-*O*-β-D-glucoside from the instrumental data as shown in Tables

Table 1. UV–Vis, IR, FAB-MS, and ^{1}H NMR Spectral Data for RP0, RP1, and BP1 Isolated from Red and Black Beans

	RP0	RP1	BP1
UV–vis λ_{max} (nm)	529.0 (log ε = 4.08) 281.0 (log ε = 3.93)	509.0 (log ε = 3.85) 432.0 (log ε = 3.32) 331.0 (log ε = 3.18) 279.0 (log ε = 3.98)	540.0 (log ε = 4.28) 279.0 (log ε = 4.11)
IR ν_{max} (cm^{-1})	3409, 1631, 1447, 1337, 1202, 1068	3364, 1609, 1443, 1339, 1201, 1064	3380, 1632, 1447, 1330, 1202, 1067
FAB-MS (*m/z*)	449 $[M]^+$	434 $[M + 1]^+$	465 $[M]^+$
^{1}H NMR (δ)	9.01 (1H, s, H4) 8.25 (1H, dd, *J* = 2.3, 9.0, H6′) 8.06 (1H, d, *J* = 2.3, H2′) 7.02 (1H, d, *J* = 8.9, H5′) 6.89 (1H, d, *J* = 2.0, H8) 6.67 (1H, d, *J* = 1.9, H6) 5.31 (1H, d, *J* = 8.0, G1) 3.95 (1H, m, G6) 3.74 (1H, m, G6) 3.51 (4H, m, G2, G3, G4, G5)	9.06 (1H, s, H4) 8.52 (2H, d, *J* = 9.2, H2′, H6′) 7.04 (2H, d, *J* = 9.2, H3′, H5′) 6.91 (1H, d, *J* = 2.0, H8) 6.66 (1H, d, *J* = 2.0, H6) 5.28 (1H, d, *J* = 7.6, G1) 3.96 (1H, m, G6) 3.73 (1H, m, G6) 3.51 (4H, m, G2, G3, G4, G5)	8.90 (1H, s, H4) 7.70 (2H, s, H2′, H6′) 6.81 (1H, br d, *J* = 2.0, H8) 6.61 (1H, d, *J* = 2.0, H6) 5.32 (1H, d, *J* = 7.6, G1) 3.94 (1H, m, G6) 3.74 (1H, m, G6) 3.60 (4H, m, G2, G3, G4, G5)

Table 2. ^{13}C NMR Spectra for RP0, RP1, and BP1 Isolated from Red and Black Beans (δ)

RP0	RP1	BP1
170.4 (s, C7)	170.8 (s, C7)	170.3 (s, C7)
164.4 (s, C2)	166.6 (s, C2)	163.7 (s, C2)
159.4 (s, C5)	164.3 (s, C5)	159.1 (s, C5)
158.3 (s, C9)	159.4 (s, C9)	157.5 (s, C9)
155.9 (s, C4′)	157.8 (s, C4′)	147.4 (s, C3′, C5′)
147.5 (s, C3′)	145.4 (s, C3)	145.8 (s, C3)
145.8 (s, C3)	137.4 (d, C4)	144.7 (s, C4′)
137.0 (d, C4)	135.8 (d, C2′, C6′)	135.9 (d, C4)
128.4 (s, C6′)	120.9 (s, C1′)	120.0 (s, C1′)
121.4 (s, C1′)	118.0 (s, C3′, C5′)	113.2 (s, C10)
118.6 (d, C2′)	113.6 (s, C10)	112.6 (d, C2′, C6′)
117.6 (d, C5′)	103.8 (d, G1)	103.6 (d, G1)
113.5 (s, C10)	103.7 (d, C6)	103.3 (d, C6)
103.9 (d, G1)	95.5 (d, C8)	95.1 (d, C8)
103.5 (d, C6)	78.8 (d, G5)	78.8 (d, G5)
95.4 (d, C8)	78.2 (d, G3)	78.1 (d, G3)
78.9 (d, G5)	74.9 (d, G2)	74.8 (d, G2)
78.2 (d, G3)	71.2 (d, G4)	71.1 (d, G4)
74.9 (d, G2)	62.5 (t, G6)	62.3 (t, G6)
71.2 (d, G4)		
62.5 (t, G6)		

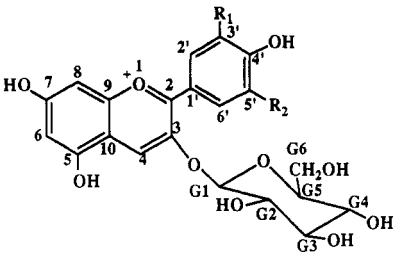
1 and 2. The ^1H NMR spectrum of the aglycon of BP1 was consistent with that of delphinidin (Toki et al., 1991), and FAB-MS spectra and UV–vis absorption (λ_{max}) were consistent with those of delphinidin 3-*O*-β-D-glucoside (Bakker and Timberlake, 1985; Saito et al., 1987). Given agreement with the literature data, the two-dimensional NMR spectra, ^1H–^1H COSY, ^{13}C–^1H COSY, HSQC (data not shown), and ^{13}C NMR spectra, BP1 was identified as delphinidin 3-*O*-β-D-glucoside.

The structures of these pigments are elucidated in Figure 2.

Antioxidative Activity of Isolated Pigments. Antioxidative activities of isolated pigments in linoleic acid system at pH 7.0 are shown in Figure 3. The pigments isolated from red and black beans, RP1 (pelargonidin 3-*O*-β-D-glucoside) and BP1 (delphinidin 3-*O*-β-D-glucoside), did not show antioxidative activity in this system at pH 7.0, but RP0 (cyanidin 3-*O*-β-D-glucoside) exhibited strong activity. The extent of activity did not significantly differ compared with that of Toc. The results measured by the TBA method showed the same tendency (data not shown).

Antioxidative Activity of Isolated Pigments in Acidic Conditions. In general, anthocyanin pigments are stable in acidic conditions (Brouillard, 1988). Igarashi et al. reported that the anthocyanin malvidin 3,5-diglucoside showed antioxidative activity in acidic conditions, suggesting the possibility that RP1 and BP1, which showed no antioxidative activity at pH 7.0, may exhibit strong activity at pH 3.0 or 5.0 (Igarashi et al., 1989). Therefore, the antioxidative activity of RP1 and BP1 in acidic conditions (pH 3.0 and 5.0) was investigated. These results are shown in Figure 4: RP1 and BP1, which showed no antioxidative activity at pH 7.0, exhibited strong activity at both pH 3.0 and 5.0. The extent of activity of RP1 and BP1 is similar to that of Toc (not significant).

Comparison of Antioxidative Activity and Stability of Pigments. Flavylium cation form of anthocyanin is stable in acidic condition (Brouillard, 1988). RP1 and BP1 showed strong antioxidative activity at pH 3.0 and 5.0 but not at pH 7.0. While these data suggest that antioxidative activity may be correlated to the stability of the flavylium cation form, RP0 showed antioxidative activity at pH 7.0, suggesting that the antioxidative mechanism of RP0 may be different from that of RP1 and BP1. Therefore, we tried to compare antioxidative activity and stability of the pigments by HPLC analysis.



R_1=OH, R_2=H Cyanidin 3-*O*-β-D-glucoside(RP0)
R_1=H, R_2=H Pelargonidin 3-*O*-β-D-glucoside(RP1)
R_1=OH, R_2=OH Delphinidin 3-*O*-β-D-glucoside(BP1)

Figure 2. Chemical structures of anthocyanin pigments isolated from red and black beans.

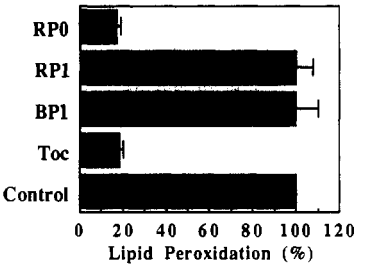


Figure 3. Antioxidative activity of isolated pigments (100 μM) from red and black beans as measured by the thiocyanate method (pH 7.0). α-Tocopherol (Toc) (100 μM) was used for the standard sample. Reported values are the mean ± SD (n = 4). A control containing no added samples or standards on its values represents 100% lipid peroxidation.

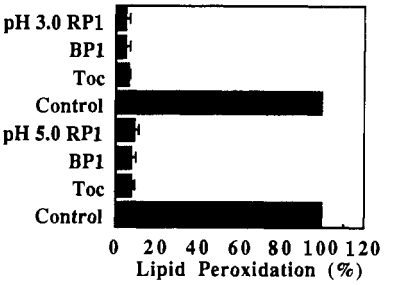


Figure 4. Antioxidative activity of isolated pigments (100 μM) from red and black beans in acidic conditions as measured by the thiocyanate method (pH 3.0 and 5.0). α-Tocopherol (Toc) (100 μM) was used for the standard sample. Reported values are the mean ± SD (n = 4). Each control containing no added samples or standards on its values represents 100% lipid peroxidation.

Figure 5 shows HPLC chromatograms of the degradation of RP0 in the linoleic acid system at pH 7.0. The HPLC peak of the pigment at UV 280 nm was very little for 4 days after incubation and completely disappeared in 10 days. However, RP0 (100 μM) showed strong antioxidative activity after the peak disappeared.

These results suggest that the antioxidative mechanism of RP0 may be different from that of RP1 and BP1. This is the first case in which an anthocyanin pigment such as cyanidin 3-*O*-β-D-glucoside exhibited strong antioxidative activity in neutral conditions. Further studies must be done on the antioxidative mechanism of the anthocyanin pigments.

Conclusion. In general, plant seed coats, such as bean and rice, contain antioxidants to protect against oxidative damage for germination during storage. There are many different color pigments in *P. vulgaris* L. seed coat, and one of the most important roles of the pigments is to protect against oxidative damage.

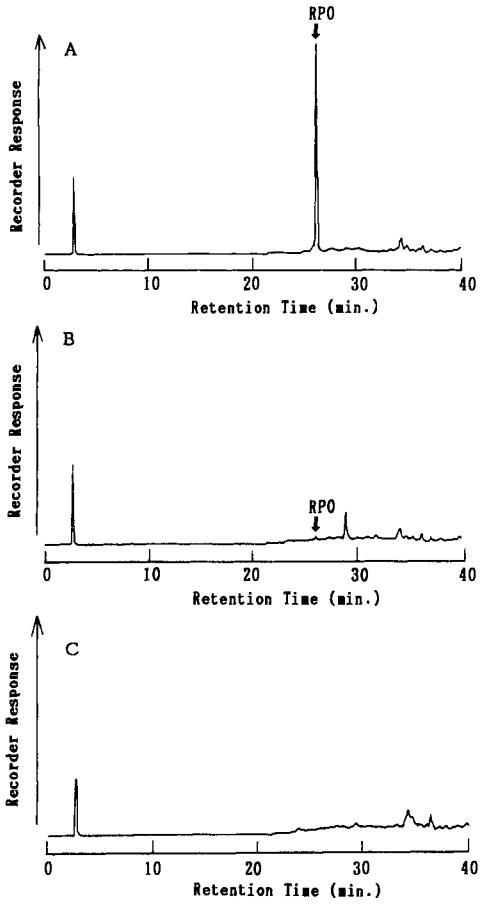


Figure 5. HPLC chromatograms of the alternations of RP0 in the linoleic acid system (pH 7.0) at intervals during incubation at 40 °C: (A) 0, (B) 4, and (C) 10 days. Conditions: column, Develosil ODS-10 (4.6 mm × 250 mm); eluent, 0.1% TFA in water; linear gradient, 0.1% TFA in acetonitrile over 30 min; flow rate, 1.0 mL/min; detector, UV 280 nm.

Pigments isolated from bean seed coats might be used not only for natural food color but also as antioxidants to increase the shelf life of foods. Anthocyanin pigments are widely distributed in the human diet of many seeds, fruits, and vegetables, indicating that we ingest many anthocyanin pigments from plant-based daily diets. Anthocyanin pigments may play an important role as dietary antioxidants after ingestion in the prevention of lipid peroxidation of cell membranes induced by active oxygen radicals in living systems. Therefore, further investigation is necessary for antioxidative activity of anthocyanin pigments *in vitro* and *in vivo*.

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