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Inhibition of Lipid Peroxidation and the Active Oxygen Radical Scavenging Effect of Anthocyanin Pigments Isolated from *Phaseolus vulgaris* L.

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ABSTRACT. No attention has been paid to anthocyanin pigments from the viewpoint of inhibitors of lipid peroxidation and scavengers of active oxygen radicals; therefore, we investigated the antioxidative, radical scavenging, and inhibitory effects on lipid peroxidation by UV light irradiation of three anthocyanin pigments, pelargonidin 3-O-β-D-glucoside (P3G), cyanidin 3-O-β-D-glucoside (C3G), and delphinidin 3-O-β-D-glucoside (D3G), isolated from the *Phaseolus vulgaris* L. seed coat, and their aglycons, pelargonidin chloride (Pel), cyanidin chloride (Cy), and delphinidin chloride (Del). All pigments had strong antioxidative activity in a liposomal system and reduced the formation of malondialdehyde by UVB irradiation. On the other hand, the extent of antioxidative activity in a rat liver microsomal system and the scavenging effect of hydroxyl radicals (·OH) and superoxide anion radicals (O_2^-) were influenced by their own structures. BIOCHEM PHARMACOL 52;7:1033–1039, 1996.

KEY WORDS. antioxidative activity; lipid peroxidation; anthocyanin; pigments; UV irradiation; radical scavenging effect

Excess production of active oxygen species, such as ·OH, ¶ O_2^-, and singlet oxygen, and other radicals are thought to cause damage in cells. This damage is believed to be strongly associated with carcinogenesis, mutagenesis, aging, and atherosclerosis [1–5]. Endogenous antioxidants are expected to protect the biological functions of cells [1, 6–9]. There is increasing interest in the protective biochemical function of natural antioxidants contained in dietary plants, which are candidates for the prevention of oxidative damage caused by oxygen free radical species [9–12].

Because no attention had been directed to edible beans as natural sources, we previously reported screening for the antioxidative activity of edible beans [13]. Among the beans tested, colored pea beans (*Phaseolus vulgaris* L.) had marked activity. These results indicated that pigments contained in the seed coat may play an important role as antioxidants. This background prompted us to investigate the biological functions of pigments chemically. Three anthocyanin pigments, P3G, C3G, and D3G, were isolated and identified as antioxidants from red and black beans (*P. vulgaris* L.) [14, 15].

Recently, the "French paradox," a low incidence of coronary heart disease and atherosclerosis despite a high-fat diet, has become evident, suggesting that phenolic compounds contained in red wine may play an important role as inhibitors of LDL oxidation [16–18]. Red wine has large amounts of anthocyanins, such as D3G, Pt3G, M3G, and C3G [19, 20], indicating that these anthocyanin pigments may contribute to the inhibitory effect of oxidation of LDL. There are few reports on biological activities, such as the antioxidative or active oxygen radical scavenging effect of anthocyanin pigments.

Anthocyanin pigments are widely distributed in the human diet through crops, beans, fruits, and vegetables [21], suggesting that we ingest considerable amounts of anthocyanin pigments from plant-based daily diets. The pigments may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems. Furthermore, they may be safe and can be used as drugs for some oxidative-damage-induced diseases.

In the present study, we evaluated the antioxidative and

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¶*Abbreviations:* ·OH, hydroxyl radicals; O_2^-, superoxide anion radicals; P3G, pelargonidin 3-O-β-D-glucoside; C3G, cyanidin 3-O-β-D-glucoside; D3G, delphinidin 3-O-β-D-glucoside; Pel, pelargonidin chloride; Cy, cyanidin chloride; Del, delphinidin chloride; Pt3G, petunidin 3-O-β-D-glucoside; M3G, malvidin 3-O-β-D-glucoside; LDL, low-density lipoprotein; Toc, α-tocopherol; AAPH, 2,2′-azobis (2-amidinopropane) hydrochloride; XOD, xanthine oxidase; Hyp, hypoxanthine; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; MDA, malondialdehyde; TBA, thiobarbituric acid; and TFA, trifluoroacetic acid.

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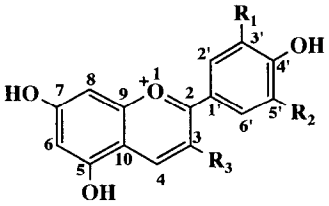
TABLE 1. Chemical structures of the anthocyanin pigments and their aglycons



Name	Substituents R_1	R_2	R_3
Pelargonidin	H	H	OH
Pelargonidin 3-O-β-D-glucoside	H	H	O-β-D-Glucoside
Cyanidin	OH	H	OH
Cyanidin 3-O-β-D-glucoside	OH	H	O-β-D-Glucoside
Delphinidin	OH	OH	OH
Delphinidin 3-O-β-D-glucoside	OH	OH	O-β-D-Glucoside

active oxygen radical scavenging activities of three anthocyanins by different systems; the structure–activity relationships of the anthocyanins are also discussed.

MATERIALS AND METHODS

Materials

Three anthocyanin pigments, P3G, C3G, and D3G, were purified from red and black bean (*P. vulgaris* L. cv. Honkintoki and *P. vulgaris* L. cv. yamashirokurosando) seed coats using HPLC. The structures and purities were confirmed by ^{1}H- and ^{13}C-NMR, FAB-MS, UV-vis, and IR spectra [14]. The purities of the three anthocyanins were more than 98% each. The aglycons of the pigments, Pel, Cy, and Del, were obtained from Extrasynthèse, Genay, France, and the purities were more than 99%. The chemical structures of the anthocyanin pigments used in the experiments are illustrated in Table 1. Egg lecithin, Toc, and AAPH were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. XOD was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Hyp was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. DMPO and DTPA were obtained from Dojindo Laboratories, Kumamoto, Japan.

Antioxidative Activity in a Liposomal System

Egg lecithin (100 mg) was sonicated in a sonicator with a 10 mM phosphate buffer (pH 7.4). The resulting multilamellar vesicles were sonicated in a cup-horn-type sonicator (Insonator 201 M, Kubota, Tokyo, Japan) at 120 W for 20 min, by which process small unilamellar vesicles were obtained. The small unilamellar vesicle solution (10 mg liposomes/mL), AAPH with a phosphate buffer (pH 7.4), and antioxidants were mixed to produce a final concentration of 1 mg liposomes/mL, 2 mM AAPH, and 1 mM phosphate buffer (pH 7.4). The test compounds were dissolved in methanol and added to the reaction mixture. The reaction mixture was stored at 37° for 4 hr, and the degree of lipid peroxidation was measured by the HPLC-urea method reported by Osawa and Shibamoto [22]. In this method, MDA was reacted with urea, and the product, 2-hydroxypyrimidine, was analyzed by HPLC. The TBA method is popular in determining lipid peroxidation levels, but this assay is not specific for MDA and is interfered with in the presence of anthocyanin pigments because the measured wavelength of the TBA reactive substance is similar to the absorption (λ_{max}) of the pigments. Therefore, we used the HPLC-urea method to determine the MDA level. The concentrations of the test compounds causing a 50% decrease in MDA formation (IC_{50} values) were based on experiments in which the compounds were tested at five different concentrations, in triplicate.

Antioxidative Activity in a Rat Liver Microsomal System

Wistar rats (8 weeks, 180–200 g) were killed, and their livers were removed and homogenized. Microsomes were prepared by differential centrifugation by the method of Slater and Sawyer [23]. Fresh solutions, in 50 mM Tris–HCl buffer (pH 7.4), were prepared each time at a concentration of 1 mg/mL microsomal protein. The test compounds were dissolved in methanol, and added to the microsomal incubation. Next, 8 mM ADP, 0.4 mM $FeCl_3$, 0.4 mM EDTA, and 0.4 mM NADPH in 50 mM Tris–HCl buffer (pH 7.4) were added (final concentrations were 2.0, 0.1, 0.1, and 0.1 mM, respectively) and incubated at 37° for 30 min according to the method of Osawa *et al.* [24], which was modified slightly. After incubation, the formation of MDA was measured using the HPLC-urea method described above. The concentrations of the test compounds causing a 50% decrease in MDA formation (IC_{50} values) were based on experiments in which the compounds were tested at five different concentrations, in triplicate.

·OH Scavenging Activity

·OH scavenging activity was evaluated by ESR spectrometry. The Fenton reaction system containing DTPA, ferrous ion, and H_2O_2 was used as the ·OH generating system, and DMPO was also used as a spin trap reagent [25]. $FeSO_4$ (340 μM), DTPA (188 μM), and DMPO (184 μM) dissolved in distilled water and the test compounds dissolved in 0.1% (v/v) TFA–water were added to 0.1 M PBS (pH 7.4). The reaction was started by the addition of 3.4 mM H_2O_2. The final concentrations of the reagents were 34 μM $FeSO_4$, 18.8 μM DTPA, and 18.4 μM DMPO; the final concentration of H_2O_2 was 340 μM. The ESR signal obtained for the DMPO–·OH adduct was measured after 1 min with a JEOL JES-RE1X spectrometer (JEOL, Ltd., Tokyo, Japan) with 100 KHz field modulation frequency. The ESR instrument conditions were as follows: microwave frequency, 9.416 KHz; power, 8 mW; field modulation width, 1.25 × 0.1 mT; time constant, 0.1 sec; receiver gain, 6.3 × 100.

Mn(II) in MnO was used as an internal standard. All experiments were done at 24°. The concentrations of the test compounds causing a 50% decrease in the relative intensity of the DMPO–·OH adduct (IC_{50} values) were based on experiments in which the compounds were tested at 3–5 different concentrations, in triplicate.

O_2^- Scavenging Activity

For the formation of O_2^-, the Hyp–XOD system was used. Hyp (5 mM), DTPA (13.8 mM) and DMPO (9.2 M) dissolved in distilled water and the test compounds dissolved in 0.1% (v/v) TFA–water were added to 0.1 M PBS (pH 7.4). The reaction was started by the addition of 1 U/mL of XOD, and O_2^- was generated enzymatically. The final concentrations of the reagents were 2 mM Hyp, 1.38 mM DTPA, 184 mM DMPO, and 0.1 U/mL XOD. The ESR signal obtained for the DMPO–O_2^- adduct was measured after 1 min. The ESR instrument conditions were as follows: microwave frequency, 9.416 KHz; power, 8 mW; field modulation width, 0.32 × 0.1 mT; time constant, 0.1 sec; receiver gain, 6.3 × 100. Mn(II) in MnO was used as an internal standard. All experiments were done at 24°. The concentrations of the test compounds causing a 50% decrease in the relative intensity of the DMPO–O_2^- adduct (IC_{50} values) were based on experiments in which the compounds were tested at 3–5 different concentrations, in triplicate.

Inhibitory Effect of Lipid Peroxidation by UV Irradiation

Liposomal solution, DTPA, phosphate buffer (pH 7.4), and antioxidants were transferred to 35 mm plastic petri dishes to produce a final concentration of 1 mg liposomes/mL, 20 μM DTPA, and 1 mM phosphate buffer (pH 7.4). The reaction mixtures were exposed to UVB at room temperature. UVB was generated by a UV health lamp (FL20S · E, TOSHIBA, Tokyo, Japan), at 1.0 mW/cm^2 at 300 nm. The lamp emits light predominantly in the UVB range but can also emit some lower and shorter wavelengths [26]. After 4 hr, the formation of MDA was measured using the HPLC-urea method described above. The concentrations of the test compounds causing a 50% decrease in MDA formation (IC_{50} values) were based on experiments in which the compounds were tested at five different concentrations, in triplicate.

Statistics

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

RESULTS AND DISCUSSION

Antioxidative Activity of Anthocyanin Pigments

Cellular membranes, which contain abundant unsaturated lipids, are a major target of free radical damage. Increased lipid peroxides are strongly associated with aging and carcinogenesis [27]. Liposomes have been used extensively as cellular models for *in vitro* lipid peroxidation studies. Therefore, we used the liposomal system for evaluating the anthocyanins as a simple *in vitro* model.

The antioxidative activity of the three anthocyanin pigments and their aglycons in the liposomal system are shown in Fig. 1. The left panel of Fig. 1 shows the inhibitory effect of the pigments on MDA formation in the liposomal system when lipid peroxidation was induced by AAPH as the radical initiator. Great inhibition of MDA formation was achieved by the addition of each anthocyanin. The formation of MDA was inhibited in a concentration-dependent manner when each anthocyanin was added. Although Toc, which is one of the endogenous antioxidants *in vivo*, was also effective, the IC_{50} values of Toc, P3G, C3G, and D3G were 7.2 ± 0.5, 1.6 ± 0.2, 1.1 ± 0.1, and 1.1 ± 0.2 μM, respectively, indicating that all anthocyanins exhibited IC_{50} values lower than that of Toc (significantly different, $P < 0.05$). P3G, C3G, and D3G had the same range of IC_{50} values (not statistically significant from each other).

The right panel of Fig. 1 shows the antioxidative activity of the aglycons (Pel, Cy, Del) in the liposomal system. Lipid peroxidation was induced by AAPH in a manner similar to that described above. All aglycons inhibited MDA formation as well as in the case of glycosides and were more effective than Toc (significantly different, $P < 0.01$). Pel, Cy, and Del had the same range of IC_{50} value (Pel: 1.7 ± 0.2, Cy: 1.1 ± 0.2, Del: 1.5 ± 0.1 μM, not significant), and these observations were the same as the results with the glycosides.

Microsomes isolated from the liver have been shown to catalyze an NADPH-dependent peroxidation of ferric ions and metal chelators, such as ADP [28]. Microsomal membranes are particularly susceptible to lipid peroxidation due to the presence of high concentrations of polyunsaturated fatty acids. We used rat liver microsomes as an enzymatic lipid peroxidation system in the presence of ADP, EDTA, and ferric ion and examined the comparative antioxidative activity of the anthocyanins.

The left panel of Fig. 2 shows the inhibitory effect of the pigments on MDA formation in a rat liver microsomal system as measured by the HPLC-urea method. D3G, which has three hydroxyl substituents in the B-ring, was the greatest inhibitor of the three anthocyanins. The IC_{50} values of D3G, C3G, and P3G were 14.1 ± 0.9, 26.3 ± 1.6, and 32.3 ± 1.5 μM, respectively, and the value of D3G was lower than that of Toc (17.2 ± 0.7 μM, significantly different, $P < 0.05$). The activity depended on the number of hydroxyl constituents of the B-ring.

The right panel of Fig. 2 shows the antioxidative activity of the aglycons (Pel, Cy, Del) and Toc in the same system. Interestingly, contrary results were obtained for the aglycons. Pel, which had only one hydroxyl substituent on the B-ring, showed the greatest antioxidative activity (IC_{50} value: 3.8 ± 0.7 μM), and the value increased in the order

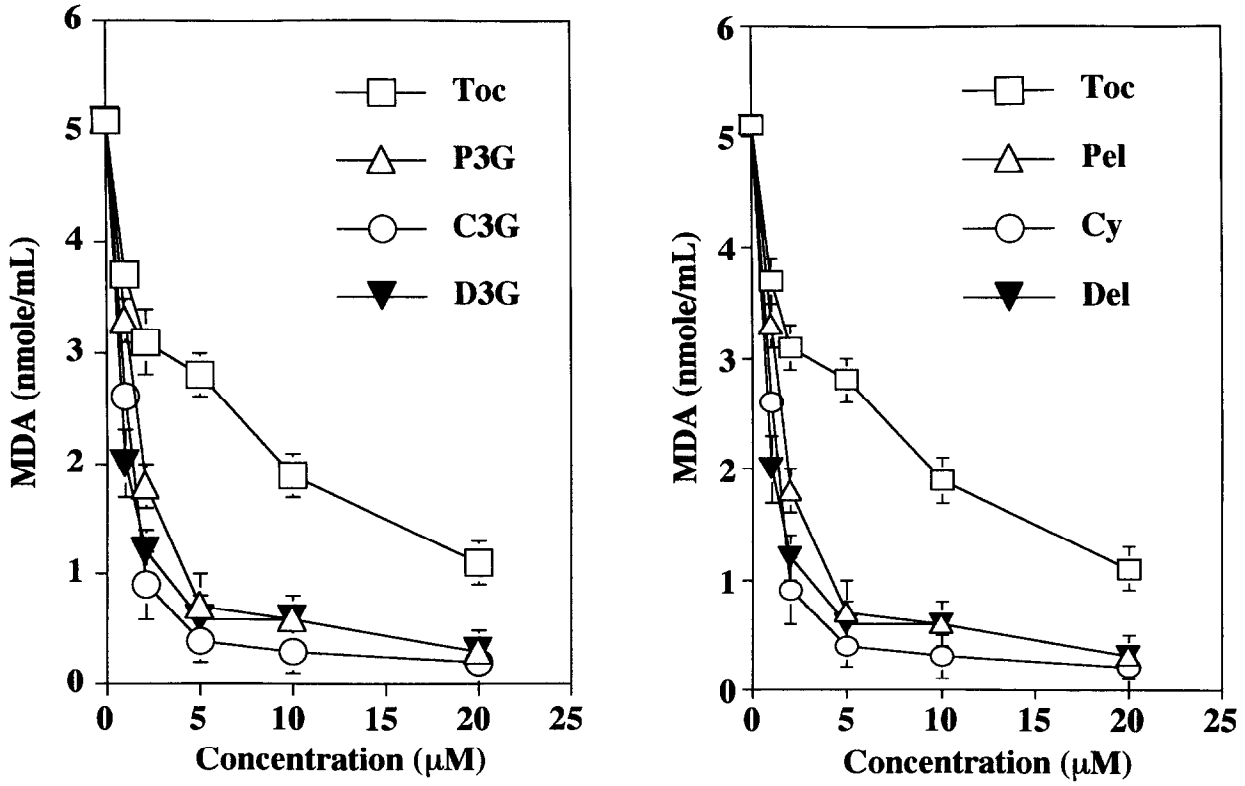


Fig. 1. Antioxidative activity of the anthocyanin pigments and their aglycons in a liposomal system. Lipid peroxidation was induced by 2 mM AAPH. Values are means ± SD (N = 3). (A) Inhibitory effects of α-tocopherol (Toc), pelargonidin 3-*O*-β-D-glucoside (P3G), cyanidin 3-*O*-β-D-glucoside (C3G), and delphinidin 3-*O*-β-D-glucoside (D3G). (B) Inhibitory effects of Toc, pelargonidin chloride (Pel), cyanidin chloride (Cy), and delphinidin chloride (Del).

of Pel < Cy (IC_{50} value: 7.7 ± 0.6 μM) < Del (IC_{50} value: 14.1 ± 0.9 μM). All aglycons had higher activity than Toc (significantly different: $P < 0.001$, compared with Pel and Cy; $P < 0.01$, compared with Del). These results indicate that as the number of hydroxyl substituents on the B-ring was increased, great activity was achieved with the glucosides, while in the case of the aglycons, increased hydroxyl substituents produced weaker activity.

Some anthocyanins may inhibit the microsomal NADPH:cytochrome P450 enzymes and result in the in-

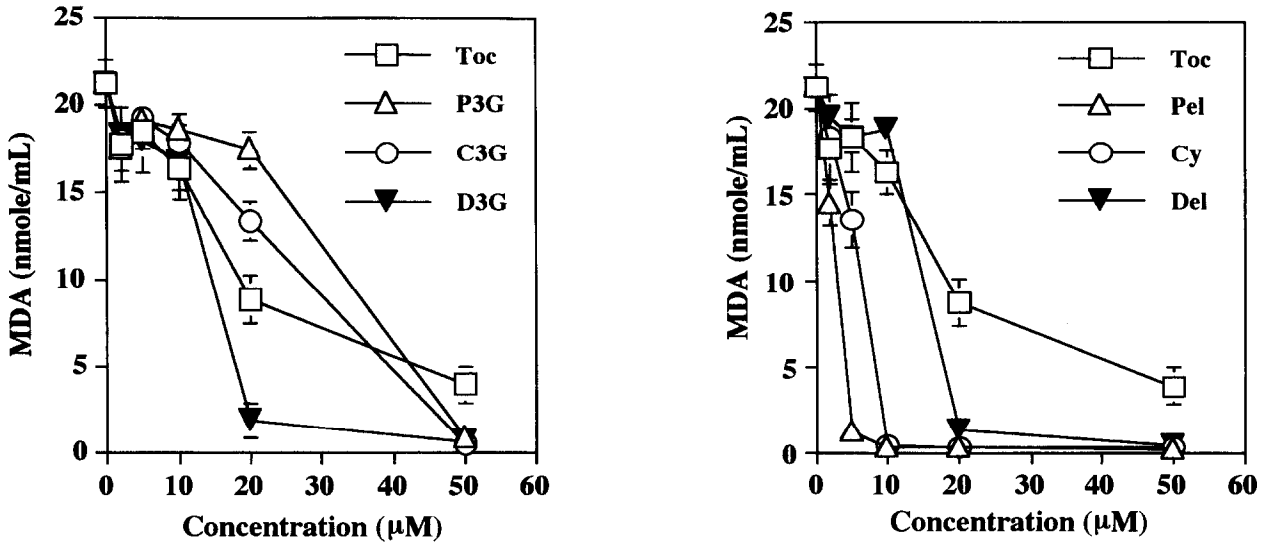


Fig. 2. Antioxidative activity of the anthocyanin pigments and their aglycons in a rat liver microsomal system. Details are given in Materials and Methods. Values are means ± SD (N = 3). (A) Inhibitory effects of Toc, P3G, C3G, and D3G. (B) Inhibitory effects of Toc, Pel, Cy, and Del. See legend of Fig. 1 for definitions of abbreviations.

hibition of MDA formation, but some of them may not inhibit them. The inhibition of the enzymes may be dependent on their structures. It would be influenced by the number of OH groups on the B-ring and glucose moiety. But in the liposomal system, lipid peroxidation is induced by AAPH or UV light, not the enzymes. This could explain why the results from the liposomal system are different from those obtained with the microsomal system.

Effect of Anthocyanin Pigments on ·OH and O_2^- Scavenging Activity

It has been revealed that active oxygen species such as ·OH and O_2^- are thought to be agents that cause oxidative damage, and much attention has been focused on active oxygen scavenging agents such as tocopherol and natural phenolics like flavonoids and tannins in preventing cell damage. Table 2 shows the active oxygen (·OH, O_2^-) scavenging activity of the anthocyanins and their aglycons. Three anthocyanins, P3G, C3G, and D3G, had the same IC_{50} values for ·OH scavenging activity (not significant). On the other hand, the aglycons, Pel, Cy, and Del, exhibited different effects. Pel was the most effective scavenger, and the activity decreased in the order of Pel > Cy > Del. These results indicate that the glucose moiety of the C-3 position contributes to weakening the ·OH scavenging activity, and the activity is dependent on the number of hydroxyl groups on the B-ring if it has no glucose moiety in any of the structures.

Husain *et al.* [29] reported that the ·OH scavenging activity of flavonoids is dependent on the number of hydroxyl groups substituted on the B-ring and of carbonyl groups at the C-4 position and that the glycosylation of the C-3 position does not alter the scavenging activity. However, our results using the anthocyanins do not reflect that of the flavonoids, indicating that the scavenging mechanism of anthocyanins may be different from that of flavonoids.

In general, the flavylium cation form of the anthocyanins is stable in the acidic condition, but the structure changes in neutral and alkaline conditions and breaks down [30]. When anthocyanins scavenge active oxygen or lipid hydroperoxide radicals, the structure also would be broken, and the radicals may be scavenged by the reaction products and show antioxidative activity. In our assay systems, the structures of the breakdown products derived from the glucosides may be different from the aglycons. The number of OH groups on the B-ring may also influence their structures. These differences, in turn, may influence the activity. Studies of the structures of the reaction products are now in progress.

TABLE 2. Effect of the anthocyanin pigments on active oxygen radical scavening activity

	·OH scavenging activity	O_2^- scavenging activity
	IC_{50} (μM)	
P3G	35.1 ± 2.5	80.3 ± 1.8
C3G	35.9 ± 2.5	12.4 ± 0.9
D3G	34.1 ± 1.6	1.6 ± 0.1*
Pel	8.5 ± 1.0	54.5 ± 6.4
Cy	36.7 ± 1.6	13.4 ± 0.7
Del	>100	2.6 ± 0.4†‡

Values are means ± SD (N = 3). Details are given under Materials and Methods* $P < 0.01$, compared with P3G and C3G.
† $P < 0.001$, compared with Pel.
‡ $P < 0.01$, compared with Cy.

The results of the O_2^- scavenging effect showed that D3G, which has three hydroxyl groups on the B-ring, was the greatest inhibitor of the three pigments. C3G also had a strong O_2^- scavenging effect; however, the extent of the activity was not stronger than that of D3G when comparing the IC_{50} values (significantly different, $P < 0.01$). On the other hand, P3G showed a weak effect. The activity of the aglycons showed exactly the same tendency as that of the glycosides. These results strongly indicate that the O_2^- scavenging effect of the anthocyanins is dependent on the number of hydroxyl groups on the B-ring. Robak and Gryglewski [31] reported that myricetin, which has three hydroxyl groups on the B-ring, showed higher O_2^- scavenging activity than quercetin, which has two hydroxyl groups on the B-ring when O_2^- was generated enzymatically using XOD [31]. These results suggest that the O_2^- scavenging mechanism of the anthocyanins is the same as that of the flavonoids.

Effect of Anthocyanin Pigments on UV Light-Induced Lipid Peroxidation

The UV light of the solar spectrum is a complex of UVA (320–400 nm), UVB (290–320 nm), and UVC (220–290 nm). Within the UV spectral region, UVB is thought to induce carcinogenesis through oxidative damage [32, 33]. When living systems are exposed to UVB irradiation, unsaturated lipids may be transformed into lipid radicals and then undergo lipid peroxidation [34]. Therefore, protection from oxidative damage caused by UV irradiation would be very important for biological systems. The liposome is the most useful *in vitro* cellular model system for the purpose of testing prevention of damage induced by UV light [35]. Therefore, we used this system in the UVB irradiation experiment.

The left panel of Fig. 3 shows the effect of the anthocyanin pigments on the formation of MDA in the liposomal system exposed to UVB. Interestingly, Toc exhibited a very weak inhibitory effect on MDA formation caused by UVB irradiation (IC_{50} value: >100 μM); on the other hand, the addition of the pigments showed a great inhibitory effect on UV-induced lipid peroxidation. The IC_{50} values of P3G, C3G, and D3G were 5.1 ± 0.3, 2.5 ± 0.1, and 0.7 ± 0.1 μM, respectively. These data suggest that the inhibitory effect is greatly associated with the number of OH substituents on the B-ring. Nishiyama *et al.* showed that Toc is a weak inhibitor of MDA levels in a lipid peroxidation system exposed to UVB [36]; they also reported that Toc is broken down rapidly by UV irradiation and becomes inactive. Therefore, Toc is a weak inhibitor when lipid peroxidation

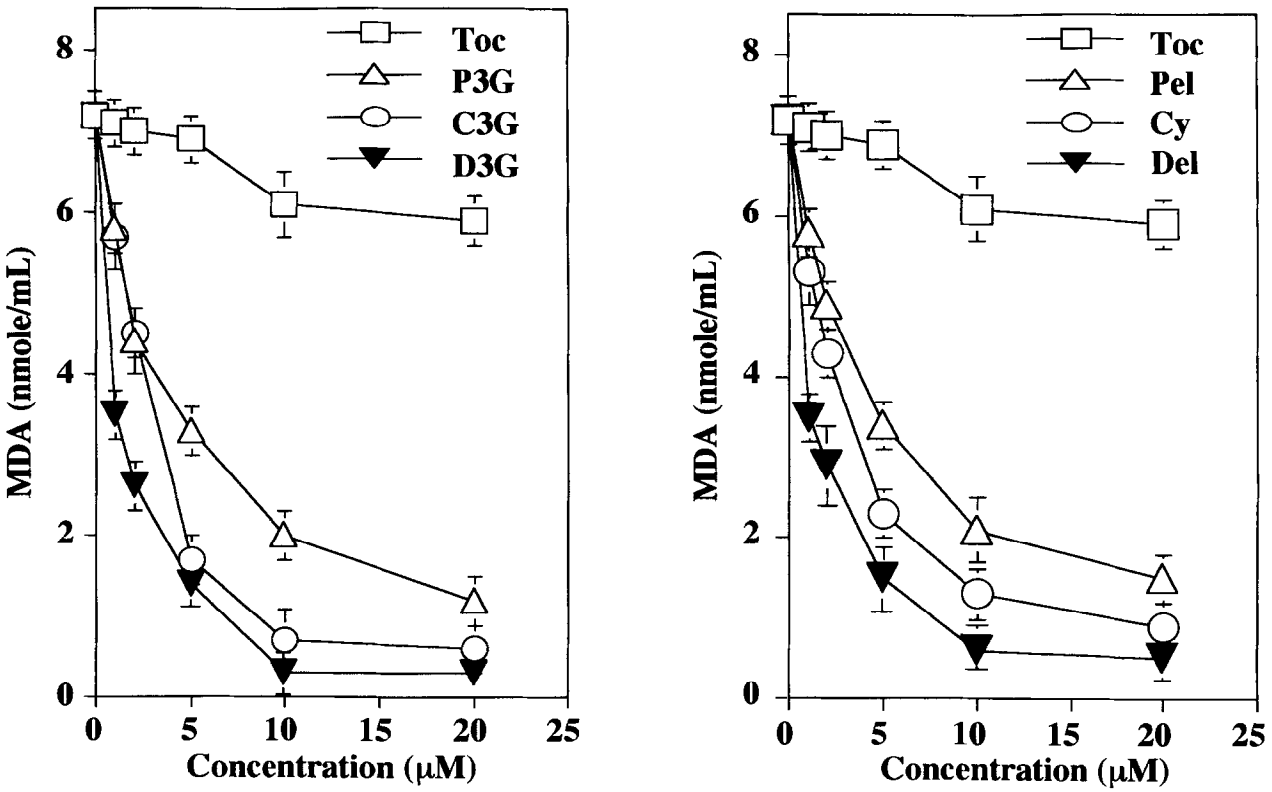


Fig. 3. Effect of the anthocyanin pigments on MDA formation in a liposomal system exposed to UVB. Details are given in Materials and Methods. Values are means ± SD (N = 3). (A) Inhibitory effects of Toc, P3G, C3G, and D3G. (B) Inhibitory effects of Toc, Pel, Cy, and Del. See legend of Fig. 1 for definitions of abbreviations.

is induced by UVB. On the other hand, the anthocyanins may also be broken down, but the reaction products by UV irradiation may retain the inhibitory effect on lipid peroxidation; a great inhibitory effect on MDA formation was achieved by the anthocyanins. The isolation and identification of the oxidation products of D3G and Toc should be investigated.

The aglycons Pel, Cy, and Del showed quite a strong inhibitory effect on the formation of MDA induced by UVB irradiation (Fig. 3, right panel), and the effect showed exactly the same tendency as that of the glycosides. The IC_{50} values of Pel, Cy, and Del were 5.3 ± 0.3, 2.4 ± 0.2, and 0.9 ± 0.1 μM, respectively. Del was the most effective inhibitor among the three aglycons (significantly different from Pel and Cy, $P < 0.01$) just as in the case of the glycosides.

Kakegawa *et al.* [37] reported that the synthesis of an anthocyanin [cyanidin 3-(6″-malonyl) glucoside] is induced by illumination with UV light in cultures of *Centaurea cyanus* cells. Takahashi *et al.* [38] also reported that anthocyanin pigment [cyanidin 3-(6″-malonyl) glucoside] is a strong inhibitor of pyrimidine dimer levels due to UV light irradiation in plant cells. These results indicate that anthocyanin pigments play a role in protecting plant cells against UV-induced damage, and may be expected to function as antioxidants to protect against UV-induced oxidative damage in animal cells.

Anthocyanin pigments are distributed widely in the human diet through seeds and crops such as fruits and vegetables. Red wine also has anthocyanins, such as M3G, Pt3G, D3G, and C3G, derived from grape skin, indicating that we ingest a lot of anthocyanin pigments in a plant-based daily diet. The results obtained from the *in vitro* assay systems are expected to play a role in reducing oxidative damage in cells.

Recently, Frankel *et al.* [39] reported that the activity of wines in protecting LDL from oxidation appears to be distributed widely among the principal phenolic compounds. Some anthocyanins are also expected to be useful for inhibition of LDL oxidation and for reducing coronary heart disease and atherosclerosis. However, there is no evidence now that anthocyanins would be effective after wine or food consumption for significantly affecting the antioxidant status of the organism. Therefore, it is necessary that the inhibitory effect of anthocyanins against oxidative damage in *in vivo* systems be investigated. We plan to carry out a feeding experiment with these anthocyanin pigments using an *in vivo* system.

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