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Superoxide radical- and peroxynitrite-scavenging activity of anthocyanins; structure-activity relationship and their synergism

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Abstract

Antioxidant activities of 15 purified bilberry anthocyanins together with pelargonidin 3-*O*-*β*-D-glucopyranoside and 4′-*O*-methyl delphinidin 3-*O*-*β*-D-glucopyranoside (MDp 3-glc), the major metabolite of delphinidin 3-*O*-*β*-D-glucopyranoside (Dp 3-glc), were evaluated in order to study the structure-antioxidant activity relationship and any synergism among them in the mixture. Both aglycone structure and the attached sugar moiety affected the $O_2^{\cdot-}$- and $ONOO^-$-scavenging activities, although the effect of the attached sugar moiety was smaller than that of the aglycone structure. The potency of activity toward the superoxide radical was in the following order: delphinidin > petunidin > malvidin ≈ cyanidin > (+)-catechin > peonidin > pelargonidin. The activity toward $ONOO^-$ was: delphinidin > cyanidin ≈ petunidin > malvidin ≈ (+)-catechin > peonidin > pelargonidin. It was confirmed that methylation of 4′-OH markedly reduced the antioxidant activity of anthocyanin. Further, it was revealed that synergism occurred in both $O_2^{\cdot-}$- and $ONOO^-$-scavenging activities among the anthocyanins in the mixture.

Keywords: *Anthocyanin, antioxidant activity, superoxide, peroxynitrite, antioxidant synergism, structure-activity relationship*

Introduction

Anthocyanins, the reddish-blue pigments present in a variety of plant tissues, are one of the major food ingredients we get from our daily diet, in the form of vegetables and red wine, as examples [1–3]. Dietary intake has been estimated at up to 200 mg/day, which is higher than that of other flavonoids such as quercetin [4,5]. Several studies have indicated the potential antioxidant property of anthocyanins [6,7]. Anthocyanins have also been reported to have many physiological functions such as vision improvement [8], and anticancer [9,10] and anti-inflammatory [11] activities.

Antioxidant activity is especially important to prevent many pathophysiological conditions [12–14]. In living systems, free radicals such as superoxide ($O_2^{\cdot-}$) and nitric oxide (NO) are associated with the pathogenesis of inflammatory diseases. Further, NO reacts with $O_2^{\cdot-}$ to produce peroxynitrite ($ONOO^-$), a highly toxic oxidizing and nitrating species toward biological molecules such as proteins and nucleic acids. 3-nitrotyrosine (3-NO_2-Tyr) is produced when $ONOO^-$ reacts with tyrosine or proteins containing tyrosine residues under physiological conditions [15]. The level of 3-NO_2-Tyr is elevated in neurodegenerative diseases, such as

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Parkinson's, Alzheimer's and amyotrophic lateral sclerosis, and therefore acts as a biomarker for oxidative stress [16–18]. Anthocyanins are considered to have potential scavenging activity against reactive oxygen and nitrogen species.

In the present study, we evaluated the scavenging activity *in vitro* of 15 purified anthocyanins from bilberry, both individually and mixed together, toward $O_2^{\cdot-}$ and $ONOO^-$, together with pelargonidin 3-*O*-*β*-D-glucopyranoside (Pg 3-glc) from strawberry and 4′-*O*-methyl delphinidin 3-*O*-*β*-D-glucopyranoside (MDp 3-glc), which is the major metabolite of delphinidin 3-*O*-*β*-D-glucopyranoside (Dp3-glc) in rat tissue. Although several studies have been published on the radical-scavenging activities of anthocyanins, they used aglycone (anthocyanidin) or fruit extract as test samples [19–27]. Moreover, only a few studies have discussed the effect of structure on antioxidant activity using a limited number of purified anthocyanins [28–32]. However, it is difficult to constract quantitative figure of the structure-activity relationship among anthocyanins from these fragmented data obtained from different laboratories and experimental conditions because anthocyanins, especially aglycones are quite labile. In the present study, using a series of purified anthocyanins, the effects of structure on the antioxidant potential of anthocyanins were comprehensively studied under the same reaction conditions in order to, for the first time, evaluate the roles of the aglycone B ring and the attached sugar moiety in their radical-scavenging activity.

Materials and methods

Chemicals

5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Labotec Co., Ltd., Japan. Xanthine oxidase (XOD); 20 U/ml, from Boehringer Mannheim Co., Germany. MCI gel CHP 20P (70–150 μm) and Sephadex LH-20 (25–100 μm) were purchased from Mitsubishi Chemicals Industries Ltd., Japan and Amarsham Pharmacia Co., Ltd., USA, respectively. All other reagents, including diethylene-triamine-*N*, *N*, *N*′, *N*′-pentaacetic acid (DTPA), L-tyrosine, 3-nitrotyrosine and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries Co., Ltd. Japan.

Bilberon 25 (powdered extract of bilberry (*Vaccinium myrtillus* L., bilberry)) was provided by Tokiwa Phytochemicals Co., Ltd. It contains fifteen types of anthocyanins (Figure 1) and the net anthocyanin content was 38% (w/w). (+)-Catechin was kindly donated by Dr Y. Kashiwada, Department of Pharmacognosy, Niigata University of Pharmacy and Applied Life Sciences.

Purification of anthocyanins

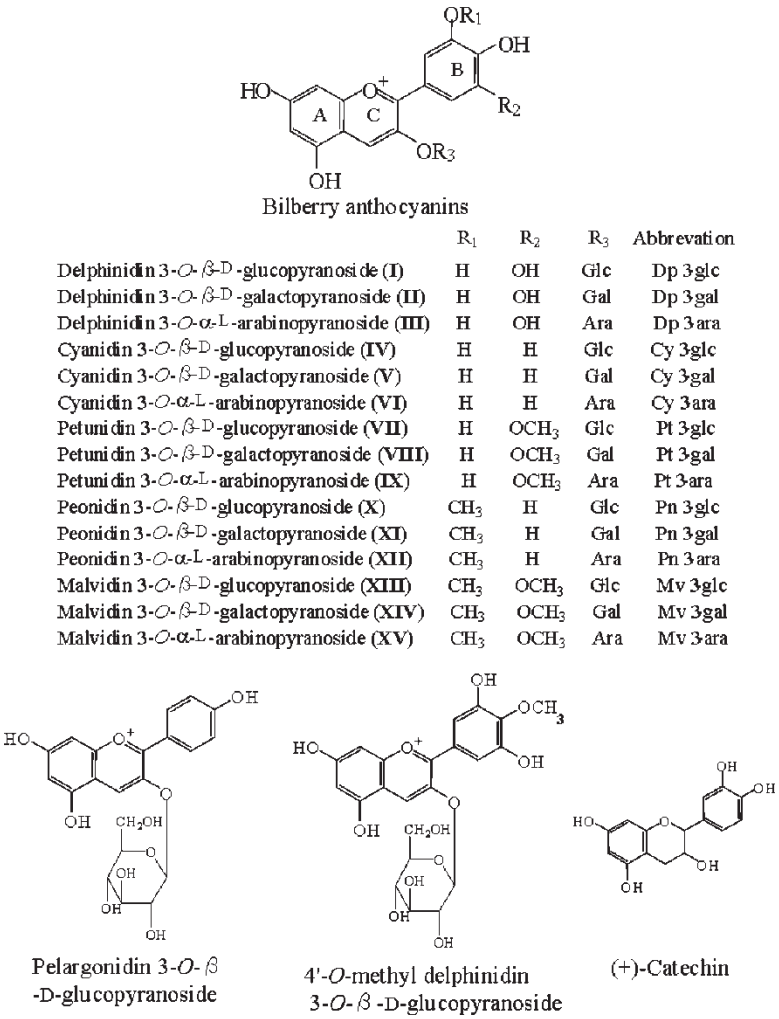
Fifteen anthocyanins from bilberry and pelargonidin 3-*O*-*β*-D-glucopyranoside from strawberry were isolated and purified using chromatographic techniques described elsewhere [33]. In brief, extraction was carried out using 1% TFA aqueous solution. Anthocyanins were recovered from an MCI column in the fraction eluted with 30% methanol (MeOH) containing 1% TFA. The anthocyanin fraction was further separated by an MCI-gel CHP (4.5 × 45 cm) column with H_2O containing increasing amounts of MeOH (0:1 → 1:0) as elution solvent to give eleven fractions. Each anthocyanin was finally purified by HPLC (Hitachi L-7120) using a Develosil ODS-HG5 column (20 × 250 mm, Nomura Chemical Co., Ltd., Japan), with 20% MeOH containing 0.1% TFA as mobile phase. All the purified anthocyanins were checked for purity and structure by tandem MS and NMR according to our previously described method [34].

Isolation and purification of the metabolite of delphinidin 3-O-β-D-glucopyranoside

The purification of MDp 3-glc was carried out according to the method described in our previous report [34]. In brief, the neck veins of male Wistar rats (8 weeks old) were cannulated using polyethylene tubes (PE-50). Dp 3-glc (dissolved in saline) was injected through the tubes into the neck veins. After 15 min, rats were sacrificed and then blood and urine were collected, and tissue (liver and kidney) was removed. Plasma and urine samples were acidified by adding 0.1 volume of 10% TFA aqueous solution and stored at −80°C until use. The liver and kidney tissue was homogenized in 3% TFA aqueous solution (0.25 g/mL), and Dp 3-glc and its metabolite were extracted with the same volume of acetonitrile. The tissue extract was combined with the plasma and urine samples, and then the solution was washed successively with dichloromethane and benzene in a 1:3 ratio. The aqueous layer thus recovered was concentrated using a rotary evaporator to remove residual organic solvent and then separated by MCI gel CHP chromatography (4.5 × 45 cm) as described above. The fractions containing MDp 3-glc were evaporated to dryness *in vacuo*, and then dissolved in 1% TFA aqueous solution for further purification by preparative HPLC using a Develosil ODS-HG5 column (20 × 250 mm).

Preparation of anthocyanin mixture

For observing the synergistic effect among anthocyanins on antioxidant activity, we prepared an anthocyanin mixture containing the 15 different anthocyanins with the same abundance as the bilberry



	R_1	R_2	R_3	Abbrevation
Delphinidin 3-*O*-β-D-glucopyranoside (I)	H	OH	Glc	Dp 3-glc
Delphinidin 3-*O*-β-D-galactopyranoside (II)	H	OH	Gal	Dp 3-gal
Delphinidin 3-*O*-α-L-arabinopyranoside (III)	H	OH	Ara	Dp 3-ara
Cyanidin 3-*O*-β-D-glucopyranoside (IV)	H	H	Glc	Cy 3-glc
Cyanidin 3-*O*-β-D-galactopyranoside (V)	H	H	Gal	Cy 3-gal
Cyanidin 3-*O*-α-L-arabinopyranoside (VI)	H	H	Ara	Cy 3-ara
Petunidin 3-*O*-β-D-glucopyranoside (VII)	H	OCH_3	Glc	Pt 3-glc
Petunidin 3-*O*-β-D-galactopyranoside (VIII)	H	OCH_3	Gal	Pt 3-gal
Petunidin 3-*O*-α-L-arabinopyranoside (IX)	H	OCH_3	Ara	Pt 3-ara
Peonidin 3-*O*-β-D-glucopyranoside (X)	CH_3	H	Glc	Pn 3-glc
Peonidin 3-*O*-β-D-galactopyranoside (XI)	CH_3	H	Gal	Pn 3-gal
Peonidin 3-*O*-α-L-arabinopyranoside (XII)	CH_3	H	Ara	Pn 3-ara
Malvidin 3-*O*-β-D-glucopyranoside (XIII)	CH_3	OCH_3	Glc	Mv 3-glc
Malvidin 3-*O*-β-D-galactopyranoside (XIV)	CH_3	OCH_3	Gal	Mv 3-gal
Malvidin 3-*O*-α-L-arabinopyranoside (XV)	CH_3	OCH_3	Ara	Mv 3-ara

Figure 1. Structure of anthocyanins and (+)-catechin. (Roman numerals correspond to the peaks in Figure 2)

extract (Table I). The cocktail of anthocyanins was made by mixing up each of the purified anthocyanins as 0.1% TFA solutions in the ratio present in the Bilberon 25. The abundance of each anthocyanin in the reconstituted mixture was checked by HPLC as shown in Figure 2.

Superoxide radical-scavenging activity measurement

Superoxide radical-scavenging activity was determined by ESR using DMPO as the spin trap reagent and a hypoxanthine-XOD system as the superoxide radical generator [35]. In this study, anthocyanin was dissolved in 0.1% TFA solution as the working stock solution, and diluted with the same solution to get appropriate concentrations immediately prior to assay. Various concentrations of anthocyanin samples or catechin as a reference antioxidant, and 0.1 U/mL XOD were added successively to the reaction mixture (total 300 μL) containing 0.2 M phosphate buffer (pH 7.8), 1 mM DTPA, 0.5 mM hypoxanthine and 33 mM DMPO. The reaction mixture was then quickly transferred into a hematocrit capillary tube. Exactly 50 s after the addition of XOD, DMPO–OOH signals were determined by ESR (JEOL JES TE

Table I. Composition of different anthocyanins in bilberry extract.

Anthocyanin	Percentage in Bilberry extract
Dp 3-glc	4.67
Dp 3-gal	4.91
Dp 3-ara	4.23
Cy 3-glc	4.12
Cy 3-gal	4.08
Cy 3-ara	3.10
Pt 3-glc	2.89
Pt 3-gal	1.51
Pt 3-ara	0.96
Pn 3-glc	1.69
Pn 3-gal	0.39
Pn 3-ara	0.24
Mv 3-glc	3.01
Mv 3-gal	1.98
Mv 3-ara	0.52
Total	38.29

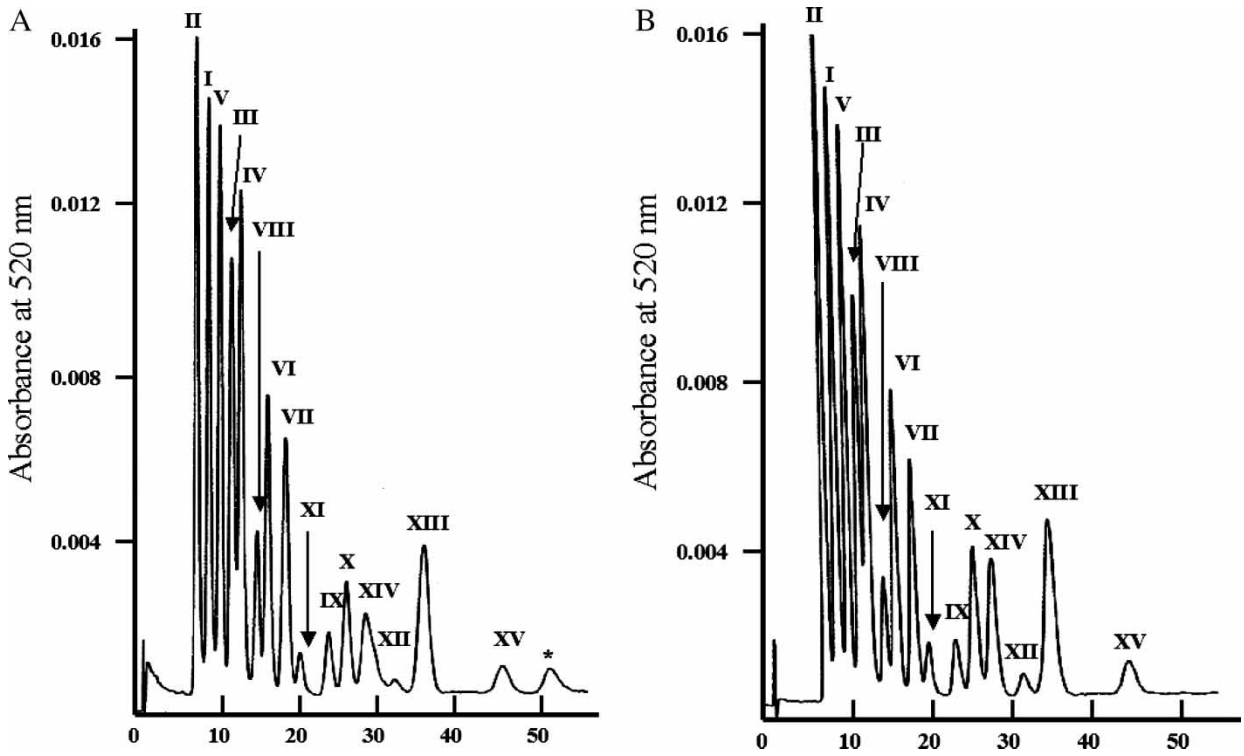


Figure 2. Typical HPLC chromatogram of anthocyanins in bilberry extract (A) and reconstituted anthocyanin mixture prepared from purified anthocyanins (B). Extra peak with (*) in figure (A) is cyanidin (aglycone), which was produced during extraction procedure.

200). The ESR settings were as follows—microwave power, 8 mW; frequency, 8.966 GHz; modulation amplitude, 0.1 mT; time constant, 0.03 s; sweep time, 30 s; center fields, 335/325 mT.

Peroxynitrite synthesis

Peroxynitrite was synthesized by the method described by Beckman et al. [36]. Acidified hydrogen peroxide (0.6 M in 0.7 M HCL, 50 mL) and sodium nitrite (0.6 M, 50 mL) in two separate syringes were simultaneously injected into ice-cooled 1.2 M sodium hydroxide (100 mL) through a Y-shaped junctor. Excess hydrogen peroxide was removed by passing the reaction solution through a manganese dioxide column. The concentration of peroxynitrite was determined by the absorbance at 302 nm ($\varepsilon = 1670\,M^{-1}\,cm^{-1}$). The typical yield of peroxynitrite ranged from 60 to 75 mM. The peroxynitrite was diluted in 0.3 M NaOH.

The inhibition of peroxynitrite-mediated tyrosine nitration by anthocyanins

The inhibition of peroxynitrite-mediated tyrosine nitration was carried out by using a modification of the method described by Rice-Evans et al. [37]. Anthocyanin was dissolved in 0.1% TFA solution as the working stock solution, and diluted with the same solution before use. Fixed concentrations of anthocyanins (100 μL) and an aliquot (5 μL) of $ONOO^-$ (10 mM) solution were added successively to 900 μL of tyrosine (300 μM) in 0.2 M phosphate buffer (pH 7) (PBS) with mixing by vortex. The final concentration of peroxynitrite in the reaction mixture was 50 μM and final concentrations of anthocyanins were 0.5–4 μM. The buffer concentration used (0.2 M phosphate) could maintain the pH of the sample solution after the addition of alkaline $ONOO^-$. Appropriate controls, without the antioxidants, were carried out to estimate the level of tyrosine nitration under the same reaction conditions.

The reactants were then analyzed by HPLC using a Develosil ODS HG-5 column (4.6 × 250 mm). HPLC was run in gradient elution mode with 50 mM PBS buffer (pH 7) and acetonitrile (MeCN) at a flow rate of 1 mL/min. The gradients used were (min/%MeCN): 0/4, 9/4, 14/60, 15/4 and 25/4. Tyrosine was monitored at 280 nm and 3-NO_2-Tyr was monitored at 420 nm, respectively. The peroxynitrite scavenging activity of the anthocyanins was calculated as the percentage decrease of 3-NO_2-Tyr formation compared to the control.

Estimating the antioxidant activity of the reconstituted mixture

For checking the synergism among anthocyanins we calculated the estimated antioxidant activity of the mixture from the scavenging activity of each of the 15

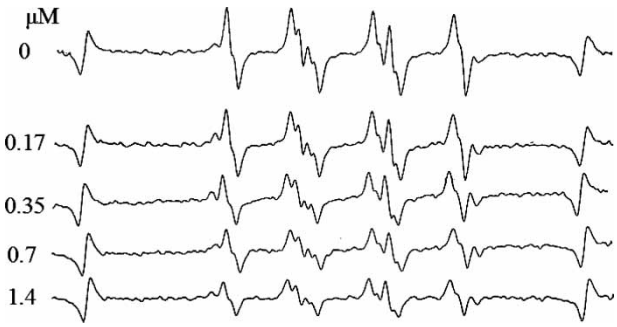


Figure 3. Typical example of superoxide anion radical-scavenging activity of Dp 3-glc.

purified bilberry anthocyanins toward $O_2^{\cdot-}$ and $ONOO^-$ as follows: using a = abundance of each component anthocyanin in Bilberon 25 and b = scavenging activity of each component anthocyanin toward either $O_2^{\cdot-}$ (IC_{30}) or 3-NO_2-Tyr formation (%), $\Sigma_1^{15} a_n b_n$ was calculated as the scavenging activity of the mixture without any synergism among the component anthocyanins.

Statistical analysis

Statistical analysis was carried out using paired and unpaired students *t*-tests. The results are expressed as means ± SEM ($n = 3$) and considered statistically significant when $p \leq 0.05$.

Results

Superoxide radical-scavenging activity

The DMPO–OOH signal produced by XO–XOD decreased in the presence of anthocyanin in a dose-dependent manner (Figure 3) due to the $O_2^{\cdot-}$-scavenging by anthocyanin. In some anthocyanin samples, however, the dose response did not extend linearly to more than 50% inhibition level, and thus the activity was compared at IC_{30} (30% inhibitory concentration) values as summarized in Figure 4. Both the number and the position of any hydroxyl substituent in the aglycone B ring, and the extent of *O*-methylation significantly affected the activity, with delphinidin showing the highest activity, followed by cyanidin. When the activity was compared among the anthocyanins having three hydroxyl substituents in the B ring (delphinidin family), it was clear that the activity decreased in relation to the extent of *O*-methylation in the B ring. MDp 3-glc, the major metabolite of Dp 3-glc, however, showed the lowest activity within its family, as well as for all the anthocyanins examined, even when only one OH group at the 4′ position was methylated. In addition it was seen that activity also changed in relation to the type of conjugated sugar, such that glucopyranoside showed higher activity than galactopyranoside, followed by arabinopyranoside (Figure 4). This trend was the same for all anthocyanins with different aglycone structures.

Tyrosine nitration

Tyrosine undergoes nitration to form 3-NO_2-Tyr when exposed to peroxynitrite at pH 7. Formation of 3-NO_2-Tyr was quantified by HPLC. Exposure of tyrosine (300 μM) to increasing concentrations of peroxynitrite (0–400 μM) resulted in an increase of 3-NO_2-Tyr production and a subsequent decrease in the levels of tyrosine (Figure 5). The total recovery of

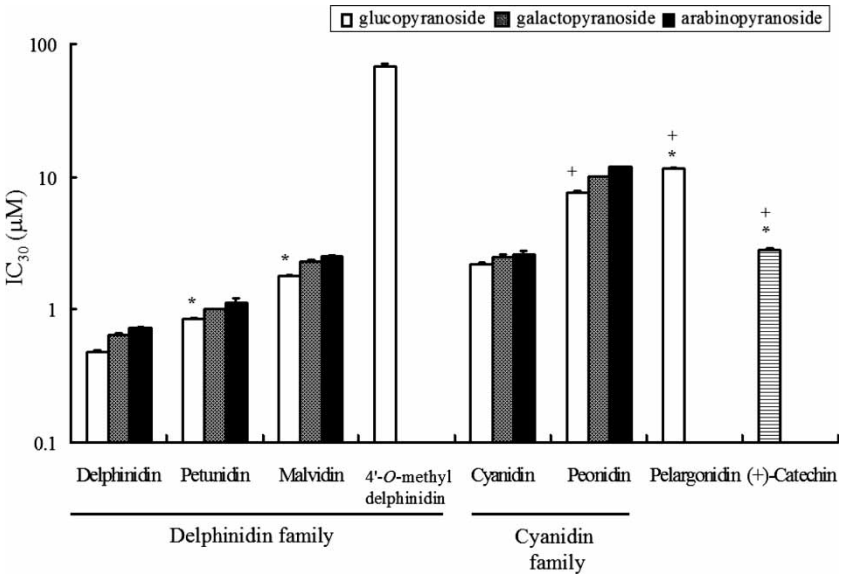


Figure 4. Superoxide-scavenging activity of anthocyanins and (+)-catechin. Values are means ± SEM of three independent experiments. *$p < 0.05$ vs. Dp 3-glc and +$p < 0.05$ vs. Cy 3-glc.

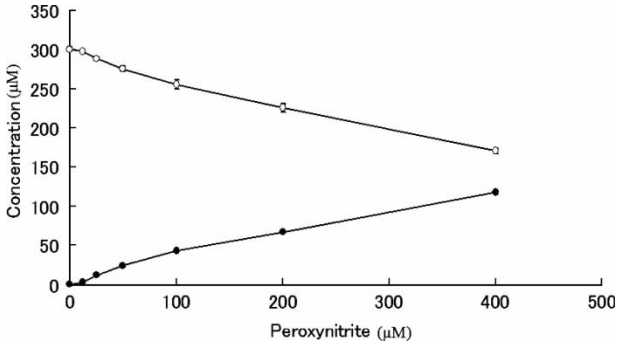


Figure 5. The extent of tyrosine nitration at increasing concentrations of peroxynitrite. Tyrosine (300 μM) in 0.2 M phosphate buffer, pH 7. Nitrotyrosine (●) and tyrosine (○).

tyrosine and 3-NO_2-Tyr in each sample was close to 100% at low concentration of peroxynitrite (10–100 μM), but decreased to 90% at higher concentration (100–400 μM). Using this method, the scavenging activity of anthocyanin toward peroxynitrite was examined (Figure 6). Results revealed that all the anthocyanins, except Pg 3-glc and MDp 3-glc, inhibited nitrotyrosine formation more strongly than catechin (Figure 6). In comparison to superoxide-scavenging activity, the peroxynitrite-scavenging activity was found less sensitive to the aglycone B ring structure although the same trend was observed in the aglycone-dependence as shown in the reactivity order (delphinidin > petunidin > malvidin).

Synergistic effect of anthocyanins

It is interesting to know whether any synergistic interaction occurs among anthocyanins when in a mixture. When we compared the observed antioxidant activity of bilberry extract (Bilberon 25) with the activity expected from the activities determined from the authentic component anthocyanins in the mixture, the observed activity was greater than that expected. Calculations of the expected activity of the mixture are given in the methods section. It was revealed that the observed scavenging activity of bilberry extract was approximately five times stronger than the estimated activity. To confirm that this enhancement of activity is due to the synergism of anthocyanins and not due to other active ingredients in the Bilberon extract, we reconstituted the Bilberon mixture from each of the purified constituent anthocyanins as shown in Figure 2. The antioxidant activity of the artificially reconstituted mixture showed almost the same level as that of Bilberon, and was approximately four times stronger than the calculated activity, indicating a certain degree of synergism occurred among the anthocyanins in the mixture in terms of antioxidant activity (Table II).

Discussion

Anthocyanins play a significant role in the antioxidant properties of fruits and vegetables, particularly those with colors. Absorption and metabolism of anthocyanin have recently been attracting much attention [34,38–42] because of their beneficial roles in health promotion. For better understanding the role of anthocyanins as antioxidants *in vivo*, structure-activity information is essential albeit for *in vitro* antioxidant properties. It is difficult, however, to discuss the structure-antioxidant relationship among anthocyanins from the fragmented data obtained under different reaction conditions from different laboratories. In the present study, we comprehensively studied the antioxidant activities of 15 purified bilberry anthocyanins together with MDp 3-glc and

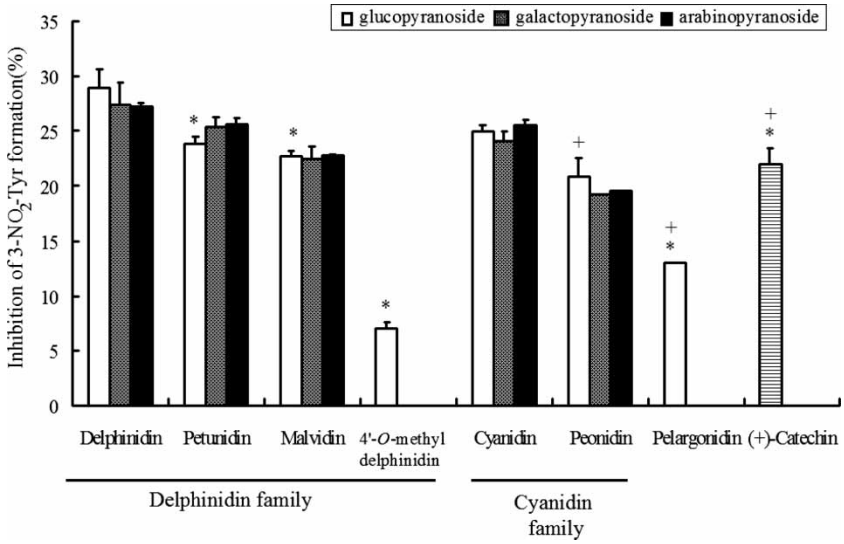


Figure 6. Peroxynitrite scavenging activity of anthocyanins and (+)-catechin compared at fixed concentration (1 μM). Values are means ± SEM of three independent experiments. $\star p < 0.05$ vs. Dp 3-glc and $+p < 0.05$ vs. Cy 3-glc.

Table II. Comparison of superoxide-scavenging activities (IC_{30} (μM)) and peroxynitrite-scavenging activities (at 1 μM concentration) of bilberry extract and the reconstituted anthocyanin mixture, and calculated estimate of activity of the mixture.

	Bilberry extract	Reconstituted anthocyanin mixture	Calculated estimate
Superoxide- scavenging activity (IC_{30} (μM))	0.17 ± 0.01	0.25 ± 0.02	0.75
Peroxynitrite- scavenging activity compared at 1 μM	42.45 ± 1.4	38 ± 2	10.3

Pg 3-glc to discuss the role of aglycone B ring structures and three types of conjugated sugar moieties in their $O_2^{\cdot-}$- and $ONOO^-$-scavenging activity. From these results, we were able to demonstrate a clear structure-activity relationship among anthocyanins toward superoxide and peroxynitrite as shown in Figures 4 and 6. For example, when activities were compared among the anthocyanins having three OH substituents in the B ring (delphinidin family), the activity was reduced as *O*-methylation in the B ring increased, regardless of the type of sugar attached. Therefore, activity ranked as follows: delphinidin > petunidin > malvidin within the delphinidin family (Figure 4). Delphinidin has three free hydroxyl groups in its B ring, whereas the 3′- and both the 3′- and 5′-hydroxyl groups were methylated in petunidin and malvidin, respectively. The same trend was observed for the anthocyanins having two OH substituents in the B ring (cyanidin family). The activity was higher in cyanidin than peonidin (Figure 4). These tendencies were also observed in the AAPH radical-scavenging activity of anthocyanins as reported elsewhere [26]. When we compare the activities of both the delphinidin and cyanidin families with those of (+)-catechin and pelargonidin, the activity was as follows: delphinidin > petunidin > malvidin ≈ cyanidin > (+)-catechin > peonidin > pelargonidin (Figure 4). Cyanidin and (+)-catechin showed comparable activity, both having a hydroxyl group at both the 3′- and 4′-positions in the B ring. It is interesting to note that petunidin and malvidin, which have methylated hydroxyl groups at the 3′-position and 3′-, 5′-positions in their B rings respectively, showed better antioxidant activity than catechin, as well as pelargonidin. This is probably because the methoxy groups in the B ring increased the stability of the resulting phenoxy radical [43,44]. Since all these analogues have a free hydroxyl group at the 4′-position in the B ring, the 4′-hydroxyl group is essential for the $O_2^{\cdot-}$-scavenging activity of flavonoids as suggested earlier [20,21]. This argument was confirmed in the present study, in which the $O_2^{\cdot-}$-scavenging activity of MDp 3-glc was examined. Moreover, as there is no anthocyanin occurring in nature with a methoxy group in the 4′-position, it was interesting to know in what way the methylation of 4′-OH affects the radical-scavenging activity. Our results clearly showed that MDp 3-glc has about 100 times less activity than that of Dp 3-glc (Figure 4) although only one methoxy group was introduced at 4′-OH indicating 4′-OH is essential for $O_2^{\cdot-}$-scavenging, as in other flavonoids, suggested elsewhere [20]. Since this metabolite was the most abundant analogue present in the tissue (liver and kidney) [34], this suggests an alternative physiological function of anthocyanins additional to that of radical-scavenger. Further research is being undertaken to clarify the physiological role of this metabolite.

We previously studied AAPH radical- and hydrogen peroxide-scavenging activities of bilberry anthocyanins [26], using capillary zone electrophoresis. The results obtained above for AAPH radical-scavenging activity were almost identical to the $O_2^{\cdot-}$-scavenging activity obtained in the present study, with Dp 3-glc the most reactive.

The effect of different sugar moieties on antioxidant activity was studied, but the results varied according to the experimental methods used [21,28,30]. Dp 3-glc and Mv 3-glc showed better activity than Dp 3-rut and Mv 3-gal, respectively. There are, however, no systematic studies on the three most commonly occurring sugar moieties in nature [21,28,30]. Therefore, it was valuable to study the role of the sugar moiety in the *in vitro* antioxidant activity of anthocyanin. In the present assay, it was revealed that the type of sugar attached affected the $O_2^{\cdot-}$-scavenging activity of anthocyanin, with glucopyranoside showing higher activity than galactopyranoside, followed by arabinopyranoside (Figure 4). These results are partially consistent with the results obtained by Kähkönen et al. [30] in which glucopyranoside showed higher activity than galactopyranoside and arabinopyranoside with cyanidin or peonidin aglycone, but there was no difference between galactopyranoside and arabinopyranoside. It has been reported that the maximal radical-scavenging activity for flavonoids is achieved if there is a free hydroxyl group in ring C [21]. We measured the $O_2^{\cdot-}$-scavenging activities of delphinidin and cyanidin aglycone and found that glycosylation at 3-OH resulted in a slight reduction of the radical-scavenging activity (data not shown).

In this study, a hypoxanthine-XOD system was used for superoxide radical generation. It has been argued that XOD is inhibited by anthocyanin as was suggested in other flavonoids [45], but recently it was confirmed that anthocyanins directly scavenged superoxide radicals even when a hypoxanthine-XOD system is used [24].

An interesting observation resulting from the present study is the effect of synergism among anthocyanins on $O_2^{\cdot-}$- and $ONOO^-$-scavenging activity. Bilberry extract (Bilberon 25) showed several times higher antioxidant activity than any single purified anthocyanin, and than the estimated activity of Bilberon mixture calculated from the activity of each component anthocyanin in the mixture. To prove this enhancement was due to the synergism among anthocyanins in the mixture rather than due to contaminated active ingredients, we reconstituted the Bilberon mixture with purified anthocyanins by mixing them with the same abundance as the bilberry extract (Table I and Figure 2). Although small differences (±5%) were observed between the reconstituted mixture and bilberry extract (Bilberon) in the Pt 3-gal (VIII), Pn 3-ara (XII) and Mv 3-gal (XIV) peaks in the chromatogram, these differences were too small to interfere with the overall activity. Although the IC_{30} (0.25 μM) for the $O_2^{\cdot-}$-scavenging activity of the reconstituted mixture was rather higher than the IC_{30} (0.17 μM) of the Bilberon 25, both IC_{30} values were several times smaller than the estimated activity (IC_{30} (0.75 μM)), indicating that synergism occurred among anthocyanins in the mixture (Table II). The same was the case for $ONOO^-$-scavenging activity, as shown in Table II. It was also suggested that the activity contributed from antioxidant ingredients other than anthocyanins, if present, was quite small compared with the antioxidant activity of the bilberry extract (Bilberon 25).

On the other hand, the aglycone-dependence of the $ONOO^-$-scavenging activity showed the same trend as the $O_2^{\cdot-}$-scavenging activity, even though the variation in activity was small among anthocyanins with different aglycones. It was, however, noted that all of the anthocyanins showed a better inhibitory effect than (+)-catechin, except for peonidin glycoside, Pg 3-glc and MDp 3-glc. It was also revealed that the type of sugar moiety did not affect the activity at all, in contrast to $O_2^{\cdot-}$-scavenging (Figure 6). The reactivity of the anthocyanins to $ONOO^-$ was in the following order: delphinidin > cyanidin ≈ petunidin > malvidin ≈ (+)-catechin > peonidin > pelargonidin (Figure 6). The $ONOO^-$-scavenging activity of MDp 3-glc was also low and about 4 times less active than Dp 3-glc, indicating 4′-OH is also important for $ONOO^-$-scavenging. It was reported elsewhere that hydroxyl groups on the B ring were nitrated when flavonoids reacted with $ONOO^-$ [23], although the nitration did not occur in the catechol B ring, as electron donation preferred to form the corresponding quinine [37,46]. We did not examine whether this reaction occurred in the present study, because the small scale of the reaction in the present study was unsuitable for the analysis of the reaction products. Further, Sies et al. [48,49] reported that flavonoids protect against peroxynitrite-dependent oxidation and nitration of tyrosine. In our present study we observed that the amount of tyrosine nitration was 100% at low $ONOO^-$ concentration (10–100 μM) but decreased to 90% at higher $ONOO^-$ concentration (100–400 μM), indicating the oxidation reaction became predominant at high $ONOO^-$ concentrations. Therefore, in the present study, the reactions were carried out at low $ONOO^-$ concentrations (50 μM). Under these conditions, the inhibitory action of anthocyanin (Dp3-glc) was also linearly correlated to the dose upto 2 μM (data not shown). Therefore, we think the nitration inhibitory activity by anthocyanins represents direct $ONOO^-$-scavenging activity.

As described in the experimental section, the reactions were carried out at neutral pH using a high concentration of buffer. A small fraction of quinoidal structure, however, was determined in the UV spectrum of the reaction mixture just before the addition of oxidants (data not shown). Since anthocyanin changes its structure at neutral pH, from the flavylium cation to carbinol pseudobase and also to the most unstable quinoidal form, further study will be needed to clarify how the structural changes of anthocyanins contribute to the antioxidant activity observed here and also *in vivo* [31,47].

In conclusion, the present study confirmed that most of the anthocyanins have better reactivity towards $O_2^{\cdot-}$ and $ONOO^-$ than (+)-catechin. Further, the aglycone structure is the primary determinant for both $O_2^{\cdot-}$- and $ONOO^-$-scavenging activity. The number of free phenolic groups on the aglycone B ring primarily determines the reactivity of anthocyanins toward free radicals. *O*-methylation of the phenolic group on the B ring reduced the reactivity of anthocyanins. In particular, *O*-methylation at the 4′-OH position was critical in reducing the scavenging activity towards both $O_2^{\cdot-}$ and $ONOO^-$. Therefore the 4′-OH group is essential for the radical-scavenging activity of anthocyanins as well as other flavonoids. Sugar moieties, on the other hand, affect $O_2^{\cdot-}$-scavenging activity to a certain extent, but not $ONOO^-$-scavenging activity. It was thus concluded that the $ONOO^-$-scavenging activity of anthocyanins was far less sensitive to structural diversity, in contrast to that toward the $O_2^{\cdot-}$ radical. It was further suggested that synergism among anthocyanins in food may play a significant role in their antioxidant properties and probably in other functions.

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