Simultaneous Comparison of Relative Reactivities of Twelve Major Anthocyanins in Bilberry towards Reactive Nitrogen Species

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The reactivities of twelve major anthocyanins identified in bilberry extracts towards nitric oxide (NO·) and peroxynitrite ($ONOO^-$) were studied *in vitro* using capillary zone electrophoresis (CZE). The reactivities of the anthocyanins towards NO· were slightly weak compared with that of (+)-catechin as a reference antioxidant under anaerobic conditions except delphinidin glycosides (Dp3glys). The reactivities of other anthocyanins were not significantly affected by either the aglycon structure or the type of sugar moiety. Under aerobic conditions, all anthocyanins and catechin showed significant enhancement of the reactivity, indicating that they reacted with other reactive species secondarily generated from NO·. Dp3glys showed rather extraordinally high reactivity towards $ONOO^-$ compared to other anthocyanins which showed approximately two times low reactivity than catechin when compared with IC_{50}. Structural divergence in the reactivity was also small for all these anthocyanins.

Key words anthocyanin; delphinidin; bilberry; structure–reactivity relationship; peroxynitrite; nitric oxide

Nitric oxide (NO·) is known as one of the key chemical mediators participating in many physiological reactions in living organisms, such as signal transduction and mitochondrial energy metabolism. However, NO· readily reacts with the superoxide anion to form a strong oxidant, peroxynitrite ($ONOO^-$), which is highly reactive to biological molecules such as proteins and nucleic acids. Peroxynitrite-mediated reactions have been extensively investigated both *in vitro* and *in vivo*, for example, nitro-tyrosine formation in proteins[1,2] and oxidation of low density lipoprotein (LDL) leading to atheroma.[3,4] Since NO· radicals are generated constitutively in the brain under physiological conditions, $ONOO^-$is implicated as a causative factor of cerebral disorders such as strokes and Alzheimer's disease.[5,6] Many polyphenols from natural origins, such as flavonol, procyanidins and catechins including epigallocatechin gallate and phenolic acids, have been studied for their scavenging activities towards $ONOO^-$ *in vitro* because purified reference samples are available.[7—12]

Anthocyanins constitute a family of flavonoids that are widely distributed in colored fruits and vegetables.[13—16] Several studies have reported on their scavenging activities against reactive oxygen and nitrogen species (ROS and RNS, respectively),[17—20] but there have been few systematic studies since the number of authentic anthocyanins available to test the reactivities is limited due to their unstable nature.

We previously determined the relative reactivities of twelve anthocyanins found in bilberries towards ROS simultaneously under the same reaction conditions using capillary zone electrophoresis (CZE).[21,22] In the present study, we further studied the reactivities of these anthocyanins towards other physiologically important reactive species, NO· and $ONOO^-$, using this CZE method, and discuss the structure–reactivity relationships among them.

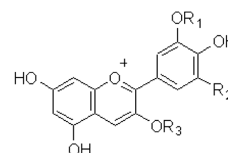
Experimental

Chemicals Na-borate ($NaBO_4$), *trans*-1,2-diaminocyclohexane *N,N,N′,N′*-tetra acetic acid monohydrate (CyDTA) and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries Co. Ltd., Japan. NOR1, a NO· generator, was purchased from Dojindo Chemical Industries Co. Ltd., Japan. CLAN K200 was obtained from Clean Chemical Co. Ltd., Japan. All other reagents were purchased from Wako Pure Chemical Industries.

$ONOO^-$ was prepared according to the method of Beckman *et al.*[23] Briefly, 0.6 M hydrogen peroxide acidified with 0.7 M hydrochloric acid was reacted with 0.6 M sodium nitrite using quenched flow equipment. The reaction was stopped with 1.2 M sodium hydroxide solution. The concentration of the $ONOO^-$ solution was determined spectroscopically to be 180 mM.

Bilberon 25 was provided by Tokiwa Phytochemicals Co. Ltd., Japan. Bilberon 25 is a powdered pure extract of bilberries (*Vaccinium myrtillus* L., bilberry) containing fifteen anthocyanins comprising five different aglycons and three different sugar moieties (Fig. 1). However, three of these (peonidin 3-*O*-α-L-arabinopyranoside, petunidin 3-*O*-α-L-arabinopyranoside and malvidin 3-*O*-α-L-arabinopyranoside) were minor components whose peaks were not detected by the CZE used in this study.[24,25] The anthocyanin content in Bilberon 25 is 33% as malvidin equivalent. (+)-catechin was kindly donated by Dr. Y. Kashiwada, Department of Pharmacognosy, Niigata University of Pharmacy and Applied Life Sciences.

Setting of Reaction Conditions for Kinetic Analysis of Anthocyanin Reactivities towards RNS To determine the appropriate conditions for studying the kinetic reactivities of the anthocyanins by CZE, the anthocyanin reactivity of Bilberon 25 was determined as a whole at various



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

Fig. 1. Chemical Structures of Bilberry Anthocyanins

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temperatures with various concentrations of NOR1 or $ONOO^-$. The reaction was followed by bleaching of the absorbance at 520 nm which was measured as an index of anthocyanidin using a HITACHI U-3000 spectrophotometer. Appropriate conditions were then determined as described in the following sections.

Conditions for the Reactions between Anthocyanins and RNS a) NO·: An aqueous solution of anthocyanin (1 mM as malvidin equivalent) was reacted with various concentrations of NOR1 (0—3 mM) as a NO· source in 0.2 M phosphate-buffered solution (PBS) at pH 7.4. The reactions were performed under both aerobic and anaerobic conditions. For the anaerobic reactions, the reaction solution was purged with argon for 20 min before the reaction, and the reaction was carried out in sealed argon-filled tube for 10 min at 37 °C. Each reaction solution was subjected to a Sep Pak C_{18} Cartridge Environment (SPE; Waters, U.S.A.) pre-equilibrated with methanol (10 ml) and 3% TFA (10 ml), and then washed successively with 10 ml of 3% TFA. Anthocyanins were recovered in the fraction eluted with 50% acetonitrile containing 1% TFA. The elution solution was evaporated to dryness *in vacuo* and dissolved again in 3% TFA aqueous solution. The supernatant was analyzed by CZE. The reference antioxidant, (+)-catechin, was reacted with NOR1 separately under the same conditions as the anthocyanin mixture.

b) $ONOO^-$: Anthocyanin aqueous solution (1 mM as malvidin equivalent) was reacted with various concentrations of $ONOO^-$ (0—0.3 mM) in 0.2 M PBS (pH 7.4) at 37 °C for 10 min. The reaction was stopped in the same manner described above and the supernatant was analyzed by CZE.

Analytical Conditions for CZE CZE was carried out in constant voltage mode at 25 °C using a CAPI-3100 capillary electrophoresis system (Otsuka Electronics Co. Ltd., Japan) equipped with a fused-silica capillary (50 μm i.d.×72.5 cm long: effective length=60.0 cm).[25] The sample solutions were loaded onto the capillary in a hydrodynamic mode (25 mm×30 s). Electrophoresis was then allowed to proceed with an average applied voltage of +25 kV. The absorption spectra were recorded at a wavelength range of 400 and 600 nm with a time constant of 0.12 s using a photodiode array detector. The electrophoretogram was recorded at 580 nm as the monitoring wavelength. The reaction of (+)-catechin was followed using a detection wavelength of 240 nm.

Results

Reactivities of Anthocyanins towards NO· Since anthocyanins reacted quite rapidly, anthocyanin mixture (Bilberon 25) was reacted with various concentrations of NOR1 or $ONOO^-$ for 10 min at 37 °C and changes of 520 nm absorbance were plotted against the concentrations. It was confirmed that the reaction occurs quantitatively with NOR1 concentration as NO· source (data not shown).

In order to confirm that neither the anthocyanins nor (+)-catechin decomposed before the reaction with RNS under the reaction conditions used, changes in the CZE peak-heights of each anthocyanin and (+)-catechin were followed for a certain time period before adding the RNS. No decreases in the peak-heights were observed, even after 15 min under the reaction conditions (in 0.2 M PBS pH 7.4, at 37 °C). Self-decomposition of both anthocyanins and (+)-catechin was, therefore, negligible under the present reaction conditions (data not shown).

Typical electrophoretograms of the anthocyanin mixture before and after the reactions with NO· under anaerobic conditions are shown in Fig. 2 (A: before the reaction; B: after the reaction with 3 mM NOR1 for 10 min at 37 °C under anaerobic conditions). The disappearance of each anthocyanin peak was plotted against RNS concentrations. From the curve obtained, IC_{50}, concentration needed to decrease 50% of 520 nm absorbance for RNS was obtained to compare the relative reactivity of anthocyanins. Typical example of kinetic treatment of the reaction is given in Fig. 3 for the case of anthocyanin reaction towards $ONOO^-$. The IC_{50} obtained for the reactions between NO· and the twelve different anthocyanins are summarized in Table 1. The reactivities towards NO· were not significantly different among all the anthocyanins under anaerobic conditions although, delphinidin 3-glycosides (Dp3glys) showed slightly higher reactivity.

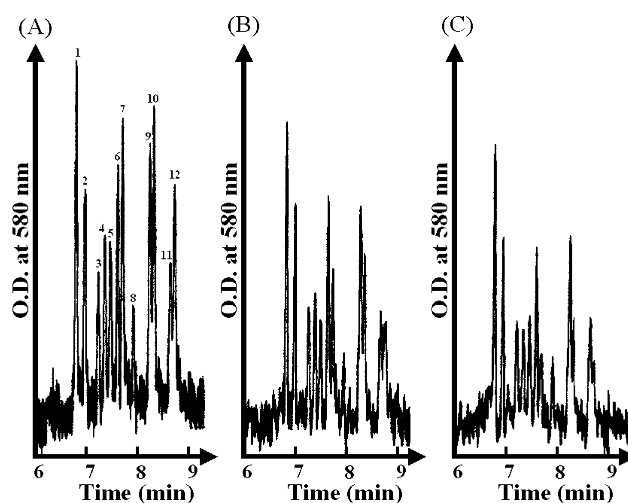

Fig. 2. Changes in the Electrophoretogram of Anthocyanins after Reaction with Reactive Nitrogen Species

The electrophoresis was carried out using 30 mM Na-borate containing 7.5 mM *trans*-1,2-diaminocyclohexane *N,N,N′,N′*-tetra acetic acid monohydrate as a carrier buffer. After each run, the capillary was washed sequentially with CLEAN99K200, distilled water, 0.5 M NaOH and distilled water for 2 min each, and then finally with running buffer. (A) before the reaction; (B) after 10 min reaction with 3 mM NOR1 under anaerobic conditions; (C) with 0.3 mM $ONOO^-$. The electrophoretograms were monitored at 580 nm. The peaks identified are as follows: 1: malvidin 3-*O*-β-D-glucopyranoside (XIII); 2: peonidin 3-*O*-β-D-glucopyranoside (X); 3: malvidin 3-*O*-β-D-galactopyranoside (XIV); 4: petunidin 3-*O*-β-D-glucopyranoside (VII); 5: peonidin 3-*O*-β-D-galactopyranoside (XI); 6: cyanidin 3-*O*-β-D-glucopyranoside (IV); 7: delphinidin 3-*O*-β-D-glucopyranoside (I); 8: petunidin 3-*O*-β-D-galactopyranoside (VIII); 9: cyanidin 3-*O*-β-D-galactopyranoside (V); 10: delphinidin 3-*O*-β-D-galactopyranoside (II); 11: cyanidin 3-*O*-α-L-arabinopyranoside (VI); 12: delphinidin 3-*O*-α-L-arabinopyranoside (III).

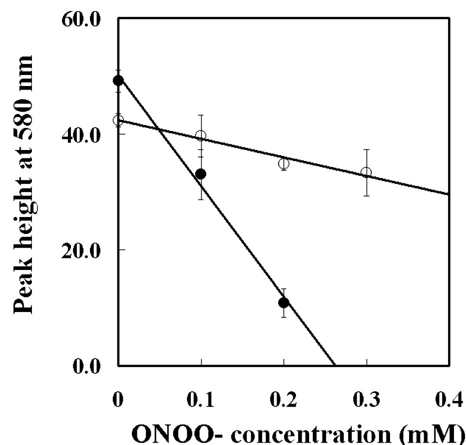

Fig. 3. Concentration Dependent Profile of $ONOO^-$ Mediated Degradation Reactions of Cyanidin 3-*O*-β-D-Glucopyranoside (IV) and Delphinidin 3-*O*-β-D-Glucopyranoside (I)

Symbols: ○; cyanidin 3-*O*-β-D-glucopyranoside (IV), ●; delphinidin 3-*O*-β-D-glucopyranoside (I).

The reactions rates of anthocyanins towards NO· occurred more significantly when the reaction was carried out under aerobic conditions, as shown in Table 2. It was also notable that Dp3glys reacted more markedly than other anthocyanins such as cyanidin 3-glycosides (Cy3glys) (Not significantly

Table 1. Relative Reactivities of Anthocyanins towards NO· under Anaerobic Conditions

	Cyandin	Peonidin	Delphinidin	Petunidin	Malvidin	(+)-Catechin
Glucoside	3.35±0.92	2.61±0.68	2.09±0.75	2.60±0.86	2.60±0.45	1.45±0.74
Galactoside	2.85±0.52	3.41±1.22	1.89±0.17	5.42±0.74	3.03±0.51	
Arabinoside	2.78±0.89	—	1.02±0.91	—	—	

Values represent the mean±S.D. of three independent determinations. Statistical comparisons were made by Student *t*-test. IC_{50} as NOR1 (mM)

Table 2. Relative Reactivities of Anthocyanins towards NO· under Aerobic Conditions

	Cyandin	Peonidin	Delphinidin	Petunidin	Malvidin	(+)-Catechin
Glucoside	2.89±0.43	2.25±0.32	1.27±0.21	2.09±0.27	1.95±0.23	1.01±0.08
Galactoside	2.31±0.44	2.44±0.38	1.28±0.03	3.34±1.13	2.83±1.47	
Arabinoside	2.42±0.23	—	1.30±0.02	—	—	

Values represent the mean±S.D. of three independent determinations. Statistical comparisons were made by Student *t*-test. IC_{50} as NOR1 (mM)

Table 3. Relative Reactivities of Anthocyanins towards $ONOO^-$

	Cyandin	Peonidin	Delphinidin	Petunidin	Malvidin	(+)-Catechin
Glucoside	0.69±0.16	0.64±0.23	0.09±0.01	0.59±0.08	0.62±0.22	0.29±0.09
Galactoside	0.59±0.15	1.63±0.74	0.10±0.01	0.76±0.17	0.61±0.02	
Arabinoside	0.66±0.21	—	0.11±0.03	—	—	

Values represent the mean±S.D. of three independent determinations. Statistical comparisons were made by Student *t*-test. IC_{50} as $ONOO^-$ (mM)

different between catechin and Dp 3-glc. Significantly different between Dp and Cy both in their galactoside and arabinoside ($p<0.05$)). These results suggest that other reactive species secondary generated from NO· under aerobic conditions might have contributed to the reactions.

Reactivities of Anthocyanins towards $ONOO^-$ The reactivities of the anthocyanins towards $ONOO^-$ were also precisely determined by the same method described above. Electrophoretograms of the anthocyanin reaction mixture before and after the reaction with $ONOO^-$ are shown in Fig. 2C. As shown in Table 3 and Fig. 2C, only Dp3glys showed remarkably high reactivities towards $ONOO^-$ compared to those of the other anthocyanins and (+)-catechin (significantly different between Dp3-glys and other anthocyanins, and catechin ($p<0.01$ or 0.05)). The reactivities of all other anthocyanins examined were approximately two times weaker than that of (+)-catechin (Table 3).

Discussion

Recently, anthocyanins have attracted much attention due to their wide range of physiological functions.[26—32] In addition, there have been several reports on the antioxidant activities of anthocyanins both *in vitro* and *in vivo*.[33] However, these reports only studied a limited number of authentic anthocyanins, and moreover, studied different reaction conditions. Since anthocyanins that have a characteristic flavylium cation structure are known to be very labile and also change their reactivity depending on the environmental pH and other factors, it is usually difficult to compare the reactivity of different anthocyanins determined independently under different conditions. Therefore, it is important to compare the reactivities of different anthocyanins under the same reaction conditions. Simultaneous comparison of the antioxidant reactivities of different anthocyanins will provide useful information for elucidating the antioxidant properties of colored fruits and vegetables showing diverse spectra of anthocyanin contents. We previously developed a CZE method for simultaneous determination of the major anthocyanins in bilberries[24,25] and studied their relative reactivities towards the superoxide anion and the hydroxyl radical.[21] It was revealed that anthocyanins had comparable scavenging activities towards the hydroxyl radical to that of (+)-catechin as a reference antioxidant, but their reactivities towards the superoxide anion were approximately 20 times higher than that of (+)-catechin. Furthermore, the reactivities towards the hydroxyl radical were not very sensitive to the structural diversity of the anthocyanins while the reactivities towards the superoxide anion were highly sensitive to the structural diversity.

In the present study, we further examined the reactivities of anthocyanins towards NO· and $ONOO^-$ which are other physiologically important reactive species. Although only a few studies have reported on the reactivities of anthocyanins with RNS, such as pelargonidin, the aglycon of strawberry anthocyanin,[34] no studies have been performed on the anthocyanins in their naturally occurring forms, that is, anthocyanoside forms. The present study showed that anthocyanins have high reactivities to RNS as well as to ROS. It was further revealed that the reactivities towards NO· were less sensitive to structural differences among the anthocyanins, that is, the reactivities were almost the same for the twelve major anthocyanins determined in bilberry, indicating that the free hydroxyl groups on the anthocyanin B ring (or the extent of methylation of these hydroxyl groups) may not contribute critically to the reactivities of anthocyanins towards NO·. This is the same trend as previously observed for the reactivities of anthocyanins towards the hydroxyl radical.[21]

The present study revealed that the reactivities of anthocyanins towards NO·, especially those of Dp3glys, were markedly enhanced under aerobic conditions (Table 2). This indicates that anthocyanins, especially delphinidins, readily react with extended oxidation products of NO·.

An interesting and important observation obtained in the present study is the uniquely high reactivities of Dp3glys to $ONOO^-$ (Table 3). The reactivities of Dp3glys were six times higher than those of the other anthocyanins although the other anthocyanins showed similar levels of reactivity towards $ONOO^-$, even with different B ring structures. Furthermore, the Dp3glys reactivities towards $ONOO^-$ were three times higher than that of (+)-catechin which is a well known $ONOO^-$ scavenger[12] (Table 3).

Although the reactivities *in vitro* is not always reflect the physiological functions, the present finding that Dp3glys have extraordinarily high potential for reacting with $ONOO^-$ suggests that delphinidin might function as stronger physiological $ONOO^-$ scavenger than cyanidin 3-*O*-*β*-D-glucopyranoside which was recently shown to have a protective effect against $ONOO^-$ induced endothelial dysfunction.[35]

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