Kinetic Comparisons of Anthocyanin Reactivities towards 2,2'-Azobis(2-aminodipropene) (AAPH) Radicals, Hydrogen Peroxide and tert-Butylhydroperoxide by Capillary Zone Electrophoresis

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Twelve major anthocyanins identified in bilberry extracts were studied in vitro using capillary zone electrophoresis (CZE) for their reactions towards 2,2'-azobis(2-aminodipropene) (AAPH) radicals, hydrogen peroxides (H$_2$O$_2$) and tert-butylhydroperoxides (t-BuOOH). Reactivity towards AAPH radicals was primarily determined by the aglycon structure, not by the type of sugar moiety. Delphinidins carrying three-hydroxyl groups on the B ring were most reactive followed by cyanidins, with two-hydroxyl groups. Further, methylation of the hydroxyl groups reduced reactivity towards AAPH radicals. However, reactivity of anthocyanins towards H$_2$O$_2$ was not significantly affected by aglycon structure or by the type of sugar moiety; there being no marked difference in reaction rates among the anthocyanins. Reactivity towards t-BuOOH was essentially the same as towards H$_2$O$_2$, although the reaction rate was several times smaller. Also, the reaction rate of anthocyanins towards peroxide was relatively high compared to that of (+)-catechin (approximately 30 times larger) measured as a reference antioxidant, whereas the reactivities of anthocyanins and (+)-catechin towards AAPH radicals were similar.

Key words anthocyanin; capillary zone electrophoresis; bilberry; structure–reactivity relationship; reactive oxygen species; hydroxyl radical

Antioxidant food ingredients have attracted attention as preventive factors against many diseases. Polyphenols, including flavonoids for example, catechins from green tea, which have been reported to be one of the strongest radical scavengers of peroxynitrite and other radicals. Their pharmacokinetic behavior has also been extensively examined. It is therefore generally accepted that flavonoids are a major food sourced antioxidant.

Anthocyanins are a kind of flavonoid widely distributed in colored fruits and vegetables such as eggplants, red cabbages, grapes and blueberries. As with other flavonoids, anthocyanins have been discussed in relation to a wide range of physiological functions such as improvement of vision, prevention of cancer and generally together with their antioxidant activity.

For example, Noda et al. studied the hydroxyl radical and superoxide radical scavenging activities of nasunin using ESR-spin trapping methods and revealed that delphinidin, the aglycon of nasunin, chelates iron ions to prevent hydroxyl radical generation during the Fenton reaction. Whilst Tsuda et al. reported the antioxidant effects of cyanidin 3-O-β-D-glucopyranoside (Cy3G) both in vivo and in vitro. Several other studies indicate the potential antioxidant properties of anthocyanins whilst the absorption and metabolic properties of anthocyanins have recently attracted much attention. However, only a few systematic studies have investigated the structure–activity relationship of anthocyanins in terms of their reactivity towards reactive oxygen species (ROS), possibly because there is limited availability of anthocyanin standards.

In the present study, we simultaneously determined the reactivities towards ROS of twelve major anthocyanins present in a wild berry extract by capillary zone electrophoresis (CZE) and discussed the structure–reactivity relationship for anthocyanins towards 2,2'-azobis(2-aminodipropene) dihydrochloride (AAPH) radicals, hydrogen peroxide (H$_2$O$_2$) and tert-butylhydroperoxide (t-BuOOH).

Experimental

Materials All reagents including trifluoroacetic acid (TFA), AAPH and H$_2$O$_2$ were obtained from Wako Pure Chemical Industries Co., Ltd., Japan. The t-BuOOH (70% aqueous solution) and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma. Bilberon 25 was donated by Tokiwa Phytochemical Co., Ltd., Japan. Bilberon 25 is the powdered pure extract of bilberry (Vaccinium myrtillus L., bilberry) containing fifteen anthocyanins with five different aglycon structures and three different type of sugar moiety (Fig. 1). The anthocyanin content in Bilberon 25 is 33% as malvidin equivalent. (+)-catechin was donated by Dr. Y. Kashiwada of the Department of Pharmacognosy, Niigata University of Pharmacy and Applied Life Sciences.

Methods Setting up of the Reaction Conditions for Kinetic Analysis of Anthocyanin Reactivity towards ROS To determine the appropriate conditions under which to study the kinetic reactivity of anthocyanins using CZE, their reactivity as a whole in the bilberry extract (Bilberon 25) was determined at various pHs and temperatures with various concentrations of AAPH radicals, H$_2$O$_2$ and t-BuOOH. Using a Hitachi U-3000 spectrophotometer the bleaching of absorbance at 520 nm was measured and deter-
mined as an index of the anthocyanin. Appropriate conditions were then determined as described in the following sections.

**Conditions for Studying the Reactivity of Anthocyanin and ROS**

(a) AAPH Radicals: The reactions of an anthocyanin aqueous solution (1 mM as malvidin equivalent) and 100 mM of AAPH at pH 5.6 (0.1 M phosphate buffer) were examined for a defined period at 50 °C during which time AAPH radicals were produced through thermolysis. The reactions were observed under aerobic and anaerobic conditions. For the anaerobic reactions, the solvent was purged with argon for 20 min before the reaction and the action was carried out in sealed tube. The final concentration of AAPH radicals (RI) was then calculated from the original AAPH concentrations used for the reaction according to Niki et al.\(^39\). The reactions were stopped by first adding 3% TFA to the solution, then cooled with ice and centrifuged. The supernatant was analyzed by the CZE method. As a reference, the reaction of the antioxidant (+)-catechin was examined independently under the same conditions as the anthocyanin mixture.

(b) \(\text{H}_2\text{O}_2\): The reactivity of anthocyanin aqueous solution (1 mM as malvidin equivalent) with \(\text{H}_2\text{O}_2\) was examined at 50 °C for a defined period in the presence of 1 mM diethylenetriamine-\(N\cdot N\cdot N\cdot N\cdot N\cdot N\cdot\)-pentacetic acid monohydrate (DTPA) or ferrozone at pH 5.6 (0.1 M phosphate buffer). Since anthocyanin reacts rapidly with \(\text{H}_2\text{O}_2\), reactivity was determined using 5 mM \(\text{H}_2\text{O}_2\). The reaction was stopped with the addition of 1000 units of catalase followed by dilution with 3% TFA. After centrifugation the supernatant was subjected to CZE analysis.

**Analytical Conditions of the CZE Method**

CZE was carried out with a constant voltage at 25 °C using a CAPI-3100 capillary electrophoresis system (Otsuka Electronics Co., Ltd., Japan) equipped with a fused-silica capillary (50μm i.d.×72.5 cm long: effective length =60.0 cm).\(^37\) The sample solutions were loaded onto the capillary with a hydrodynamic mode (30 s). Electrophoresis was then allowed to run with an average applied voltage of +30 kV. The absorption spectra were recorded at a wavelength range of 400 to 600 nm with a time constant of 0.12 s using a photodiode array detector. Electrophoretograms were recorded at 580 nm by monitoring the wavelength. For the analysis of (+)-catechin, the detection wavelength and applied voltage were set at 240 nm and +25 kV, respectively.

**Results**

**Reaction of Anthocyanin towards AAPH Radicals**

First, self-decompositions of anthocyanins at various pHs were studied. Anthocyanins were quite unstable, decomposing rapidly at neutral and alkaline pHs. Therefore, the reactions were carried out at pH 5.6. To examine the heat stability of the anthocyanins and (+)-catechin, they were kept in the reaction buffer at 50 °C without adding AAPH, and the peak-height changes were followed by CZE; but no significant decomposition was observed even after 120 min of the reaction. Heat decomposition of both the anthocyanins and (+)-catechin was, therefore, negligible under the present reaction conditions (data not shown).

The bleaching rate of the anthocyanin mixture towards AAPH radicals under anaerobic conditions was approximately half the magnitude of the rate seen under aerobic conditions (data not shown). It was thus suggested that oxygen derived secondary species such as peroxylradicals were involved in the accelerated decomposition of anthocyanin under aerobic conditions. To avoid this complexity, the following experiments were carried out under anaerobic conditions. Typical electrophoretograms of the anthocyanin mixture before and after the reactions with AAPH are shown in Figs. 2A and B. Changes in each anthocyanin peak were plotted against the reaction time in a logarithmic scale. The results showed that the decreasing rate of CZE peaks was followed, the pseudo-first order kinetics as shown in Fig. 3. When the reaction rates of anthocyanins with the same aglycon were compared, the reaction rates were similar among them (Fig. 3A). The reaction rates among anthocyanins carrying the same sugar moiety were also compared (Fig. 3B).

In this case, the reaction rates were dependent on the aglycon structure; such that delphinidin glycosides disappeared faster than any other anthocyanins. Thus the aglycon structure is a major determinant for reactivity of anthocyanins towards AAPH radicals.

The rate constants for each anthocyanin towards AAPH radicals were calculated from the slopes in Fig. 3, and are summarized in Table 1. It is clearly shown that delphinidins were more reactive than cyanidins in any glycosylated derivatives (\(p<0.01\)). Furthermore, reactivity towards AAPH radicals decreased with the extent of methylation of the hydroxyl group on the B ring. The extent of the reactivities among the
Table 1. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward AAPH Radicals (min⁻¹×10⁴)

| Anthocyanidin Glucoside Galactoside Arabinoside (+)-Catechin |
|------------------|------------------|------------------|------------------|
| Cyanidin         | 1.05±0.19        | 1.55±0.23        | 1.6±0.49         | 3.78±0.53 |
| Peonidin         | 0.85±0.31        | 1.17±0.44        | —                | —        |
| Delphinidin      | 2.77±0.43        | 3.04±0.41        | 3.22±0.23        | —        |
| Petunidin        | 1.6±0.32         | 2.35±0.49        | —                | —        |
| Malvidin         | 1.21±0.22        | 1.52±0.32        | —                | —        |

Bilberry extract (1 mS for malvidin equivalent) or 1 mS of (+)-catechin was reacted with AAPH for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin as shown in Fig. 3. Values are means±S.D. for three reactions. Difference between delphinidin and other anthocyanins (cyanidin, peonidin and malvidin) was significant in all of the glycosides (p<0.01) and also petunidin-glycoside (p<0.05). Difference between (+)-catechin and anthocyanidin (cyanidin, peonidin and malvidin) was significant in all of the glycosides (p<0.01) and also petunidin glycoside (p<0.05). Statistical comparisons were made by Student t-test.

Table 2. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward H₂O₂ (min⁻¹×10⁴)

| Anthocyanidin Glucoside Galactoside Arabinoside (+)-Catechin |
|------------------|------------------|------------------|------------------|
| Cyanidin         | 5.86±0.79        | 6.53±0.73        | 5.80±0.61        | 0.187±0.02 |
| Peonidin         | 5.74±0.39        | 4.58±0.54        | —                | —        |
| Delphinidin      | 5.2±0.88         | 5.99±0.6         | 5.37±0.51        | —        |
| Petunidin        | 4.36±0.73        | 4.74±0.66        | —                | —        |
| Malvidin         | 6.41±0.26        | 4.76±0.55        | —                | —        |

Values are means±S.D. for three reactions. Bilberry extract (1 mS for malvidin equivalent) or 1 mS of (+)-catechin was reacted with 5 mM H₂O₂ at 50 °C for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin. Values are means±S.D. for three reactions. Difference between (+)-catechin and all anthocyanin was significantly different (p<0.01). Statistical comparisons were made with Student t-test.

Table 3. Pseudo-First Order Rate Constants for the Reaction of Anthocyanins toward t-BuOOH (min⁻¹×10⁴)

| Anthocyanidin Glucoside Galactoside Arabinoside (+)-Catechin |
|------------------|------------------|------------------|------------------|
| Cyanidin         | 5.19±0.95        | 5.44±0.53        | 5.45±0.48        | 0.124±0.072 |
| Peonidin         | 4.9±0.46         | 4.18±1.14        | —                | —        |
| Delphinidin      | 4.44±0.35        | 5.11±0.5         | 5.13±0.55        | —        |
| Petunidin        | 5.45±0.92        | 4.53±0.6         | —                | —        |
| Malvidin         | 3.77±0.34        | 3.82±0.85        | —                | —        |

Bilberry extract (1 mS for malvidin equivalent), or 1 mS of (+)-catechin reacted with t-BuOOH for a defined period. After the reaction, the supernatant was subjected to CZE. The rate constants were obtained from the slopes of each anthocyanin. Values are means±S.D. for three reactions. Difference between (+)-catechin and all anthocyanin was significantly different (p<0.01). Statistical comparisons were made by Student t-test.

**Discussion**

The antioxidant properties of flavonoids, including some anthocyanins, have been studied extensively, but no systematic study has been undertaken in terms of the structure-reactivity relationship of anthocyanin reactions towards ROS. As the authors previously showed, the CZE method is useful not only to profile anthocyanin distribution in plant extracts but also to follow the kinetic changes of the contents under certain reaction conditions. In the present experiments, the reactions of twelve major anthocyanins present in a bilberry extract (Bilberon 25) towards ROS were quantitatively evaluated by CZE. The reactions with ROS were carried out at pH 5.6, not at the physiological pH, because anthocyanins are quite unstable at alkaline and neutral pHs. The temperature was kept at 50 °C, also not at the physiological temperature, to promote the reactions for an accurate determination of reaction rates. Temperature, however, did not affect the relative reactivity of anthocyanins with ROS.

Although the contents of each anthocyanin in Bilberon 25 samples are not identical, for example, malvidin 3-O-β-D-glucopyranoside presents in a quantity more than 5 times excess to petunidin 3-O-β-D-galactopyranoside, all the anthocyanin peaks decreased linearly in a log plot (See Fig. 3) during the reaction period up to 120 min; indicating that all the anthocyanins present in the sample followed pseudo-first order kinetics under the present reaction condition. Therefore, the relative reactivities of the twelve different anthocyanins were reliably compared using Bilberon 25 as the an-
When the reactivity towards AAPH radicals was compared among the bilberry anthocyanins, it was discovered that the number of hydroxyl substituents on the anthocyanin B ring primarily determined the radical scavenging ability of the anthocyanins. The methylation of these hydroxyl groups led to a significant decrease in reactivity (see Table 1). Structure–activity relationships of anthocyanins for superoxide quenching reactions have been reported. In those studies, the numbers of free hydroxyl groups on the anthocyanin B ring related directly to the scavenging activities. In this study, the reactivity of anthocyanins towards AAPH radicals showed a similar trend as that towards superoxide anion radicals. Since the methylation of these hydroxyl substituents reduced reactivity towards AAPH radicals, it is suggested that the AAPH radicals reacted to anthocyanins via hydrogen atom abstraction from the hydroxyl group(s) on the B ring as in other flavonoids.

On the other hand, the type of sugar moiety did not really affect the reactivity towards AAPH radicals. This trend is different from previous observations where acid mediated hydrolysis of the anthocyanins was shown to be governed mainly by the type of sugar moiety, not the aglycon structure.

The reactivity of anthocyanins towards \( \text{H}_2\text{O}_2 \), however, was completely different from that towards AAPH radicals. Anthocyanins again showed remarkably higher reactivity than (\(+\)-)catechin, which is known to be a strong radical scavenger. The reactivity of cyanidins was a little more than that of delphinidins, but the difference was not statistically significant. Therefore it seems the B ring structure is not critical in determining the reactivity of anthocyanins towards \( \text{H}_2\text{O}_2 \).

Although the reaction rate of (\(+\)-)catechin was not determined in the presence of Bilberon 25 because the CZE peak overlapped with other ingredients in the mixture, the reaction rate was comparable to that towards AAPH radicals, or several times smaller than those for anthocyanins towards peroxides. Since the presence of competitive reactions might affect the reaction rate of (\(+\)-)catechin towards ROS, reducing rather than increasing, it was concluded that the reactivity of anthocyanins towards AAPH radical and peroxides was at least stronger than (\(+\)-)catechin.

Some chemical studies have been done on the bleaching reaction of anthocyanin to sodium sulfite and \( \text{H}_2\text{O}_2 \) in the bilberry extract. In these studies, malvin (malvidin 3,5-O-\( \beta \)-d-diglucopyranoside) was reacted with \( \text{H}_2\text{O}_2 \) to cleave the C ring leading to malvin formation. It is suggested that the same reactions occurred here between anthocyanidins 3-O-\( \beta \)-d-monoglycoside, and \( \text{H}_2\text{O}_2 \) in this study, in that \( \text{H}_2\text{O}_2 \) attacks the 2,3-doubly bond of the anthocyanin C ring. Since (\(+\)-)catechin does not have a double bond in the 2,3-position of the C ring, the low reactivity of (\(+\)-)catechin towards peroxides can be substantiated. Another factor, which may accelerate bleaching of the anthocyanins, might be hydroxyl radicals, which must be produced by the Fenton reaction if iron is present in the bilberry extract. The iron content in Bilberon 25 is approximately 1 mg/100 g (assay data by Tokiwa Phytochemical Co., Ltd.). From this data, it was calculated that the iron concentration in the reaction mixture was less than 60 nm. Therefore, the anthocyanin decomposition reactions in the presence of excess amounts of ferrozine or DTPA (0.5 mm for ferrozine and 0.1 mm for DTPA respectively) were studied to eliminate the possible involvement of the Fenton reaction. The results clearly showed that the electrophoretogram of the reaction mixture was almost identical to that without ferrozine. It was concluded therefore, that hydroxyl radical mediated reactions were negligible in these experiments.

A question might arise that present observation is only the chemical reactivity of anthocyanins, and not their scavenging potential against ROS. However, the high reactivity of anthocyanins compared to (\(+\)-)catechin as a reference strongly suggests the high potential of their scavenging ability against ROS. Moreover, Yamasaki et al. reported that anthocyanin bleaching was accelerated when cyanidin 3-O-\( \beta \)-p-sophoroside scavenges superoxide anion radical. Clarification of the quantitative correlation between chromophore bleaching and antioxidant activity, however, has to be awaited until each anthocyanin is isolated and purified.

In conclusion, the present experiments revealed that anthocyanins readily reacted, to bleach, with AAPH radicals and hydroperoxides (\( \text{H}_2\text{O}_2 \) and t-BuOOH), but their reactivity towards peroxides was more remarkable compared to the reactions towards AAPH radicals. The reactivity of anthocyanins was mainly governed by the aglycon structure, and not by the type of sugar moiety in both reactions; for example, delphinidins were the most reactive anthocyanin towards both AAPH radicals and peroxides. These observations will be valuable for the elucidation of the antioxidant role of anthocyanins in certain food sources.

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