

## Acid Mediated Hydrolysis of Blueberry Anthocyanins

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Acid mediated hydrolysis of anthocyanins was studied using capillary zone electrophoresis (CZE). A commercially available wild blueberry (Bilberry) extract was dissolved in different concentrations of TFA (0.1, 1, 3, 9%), then was subjected to thermodecomposition reaction at 95 °C. After the reaction, the samples were analyzed by CZE. The hydrolysis rate of each anthocyanin and the formation of the aglycon were determined by the change in the peak pattern of the anthocyanins in the electropherogram. Each anthocyanin peak decreased time dependently in a first order kinetic fashion. It was revealed that the hydrolysis rate of each anthocyanin was determined primarily by the type of conjugated sugar and not by the aglycon structure. The rate constant of anthocyanin hydrolysis was in the following order, arabinoside > galactoside > glucoside without regard to the aglycon structure. The kinetic behavior of this anthocyanin hydrolysis together with the CZE mobility allowed us to identify an unknown CZE peak as delphinidin 3-*O*- $\beta$ -arabinoside. At low TFA concentration, significant decomposition of the anthocyanidin nucleus occurred, but the glycoside hydrolysis predominated at high TFA concentration. It was further revealed that the aglycon released reacted successively to form polymeric products at higher TFA conditions.

**Key words** blueberry anthocyanin; capillary electrophoresis; anthocyanin decomposition; glycoside hydrolysis

Anthocyanins are the reddish pigments widely distributed in colored fruits and vegetables, such as eggplant, grapes and blueberry. They are usually present as the glycosides having arabinose, glucose or galactose attached to the anthocyanidin nucleus. The phytochemical significance of anthocyanins has been discussed in relation to a wide range of physiological functions such as vision improvement,<sup>1)</sup> antioxidant,<sup>2–5)</sup> anticancer<sup>6)</sup> and so on. Few studies have been done on the structure–radical scavenging activity relationship among anthocyanin aglycons and the glycosides.<sup>7)</sup>

It is known that the flavylium cation form of the anthocyanidin nucleus is unstable under conditions such as neutral and alkaline pH, high temperature and light illumination.<sup>8)</sup> From the point of view of using anthocyanins as a functional food material, it is important to know the stability of anthocyanins as a mixture.

Since there are few studies on the degradation property of anthocyanins, we precisely examined here the acid mediated reaction of anthocyanins by capillary zone electrophoresis (CZE) using commercially available wild berry (Bilberry) extract as the sample of anthocyanin mixture. The results revealed that the hydrolytic rate of anthocyanin glycoside was not dependent on the aglycon structure, but dependent on the type of conjugated sugar.

### Materials and Methods

**Reagents** All the reagents including Na-borate (NaBO<sub>3</sub>), *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. CLEAN 99 K200 was from Clean Chemical Co. Ltd., Japan.

Bilberon 25, the concentrated extract of Bilberry (*Vaccinium myrtillus* L., BILBERRY), was a kind gift from Tokiwa Phytochemicals Co. Ltd.

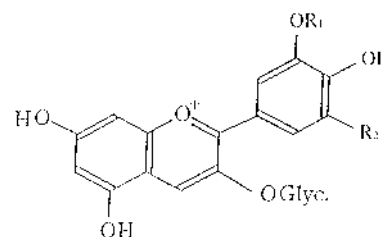
**Methods. Acid Mediated Hydrolysis of Anthocyanin** Bilberon 25 was dissolved in different concentrations of TFA (0.1, 1, 3, 9%) to make the concentration to  $6.47 \times 10^{-3}$  mol delphinidin eq/l (calculated using  $\epsilon = 30906$  at 525 nm absorbance). An aliquot was taken into an ampule, sealed with Argon, then was subjected to hydrolytic reaction at 95 °C for a defined period. After the reaction, each sample was analyzed by CZE to determine the hydrolytic degradation rate of each anthocyanin.

**Analysis of Anthocyanins by CZE** An aliquot of the above reaction so-

lution was diluted 10 times with 1% TFA, then 100  $\mu$ l of the solution was taken in a sample tube, dried up *in vacuo* and finally dissolved in 1 ml of 1% TFA. After passing through a 0.2  $\mu$ m  $\phi$  membrane filter, the sample solutions were subjected to CZE. Triplicate determinations were run for each sample. The CZE conditions were as reported previously.<sup>9)</sup>

### Results

Hydrolysis of each anthocyanin in the Bilberry extract was determined in 1% TFA using the CZE method. Twelve anthocyanin peaks were identified as shown in Fig. 2 from the comparison of the mobility of standard anthocyanins as described in our previous report.<sup>9)</sup> When the TFA solution of the Bilberry extract was heated in a sealed ampule filled with Argon at 95 °C, the CZE peaks of each anthocyanin decreased with reaction time, concomitantly with an increase of aglycon peak (Fig. 2). The time course changes of anthocyanin and the aglycon peak are shown in Fig. 3 together with the 525 nm absorbance ( $A_{525\text{nm}}$ ) change of the reaction solution that was determined as the indication of anthocyanidin nucleus. It was revealed that the aglycon formation



AGLYCON	R <sub>1</sub>	R <sub>2</sub>
DELPHINIDIN	H	OH
CYANIDIN	H	H
PETUNIDIN	CH <sub>3</sub>	OH
PEONIDIN	CH <sub>3</sub>	H
MALVIDIN	CH <sub>3</sub>	OCH <sub>3</sub>

Fig. 1. Structure of Anthocyanins

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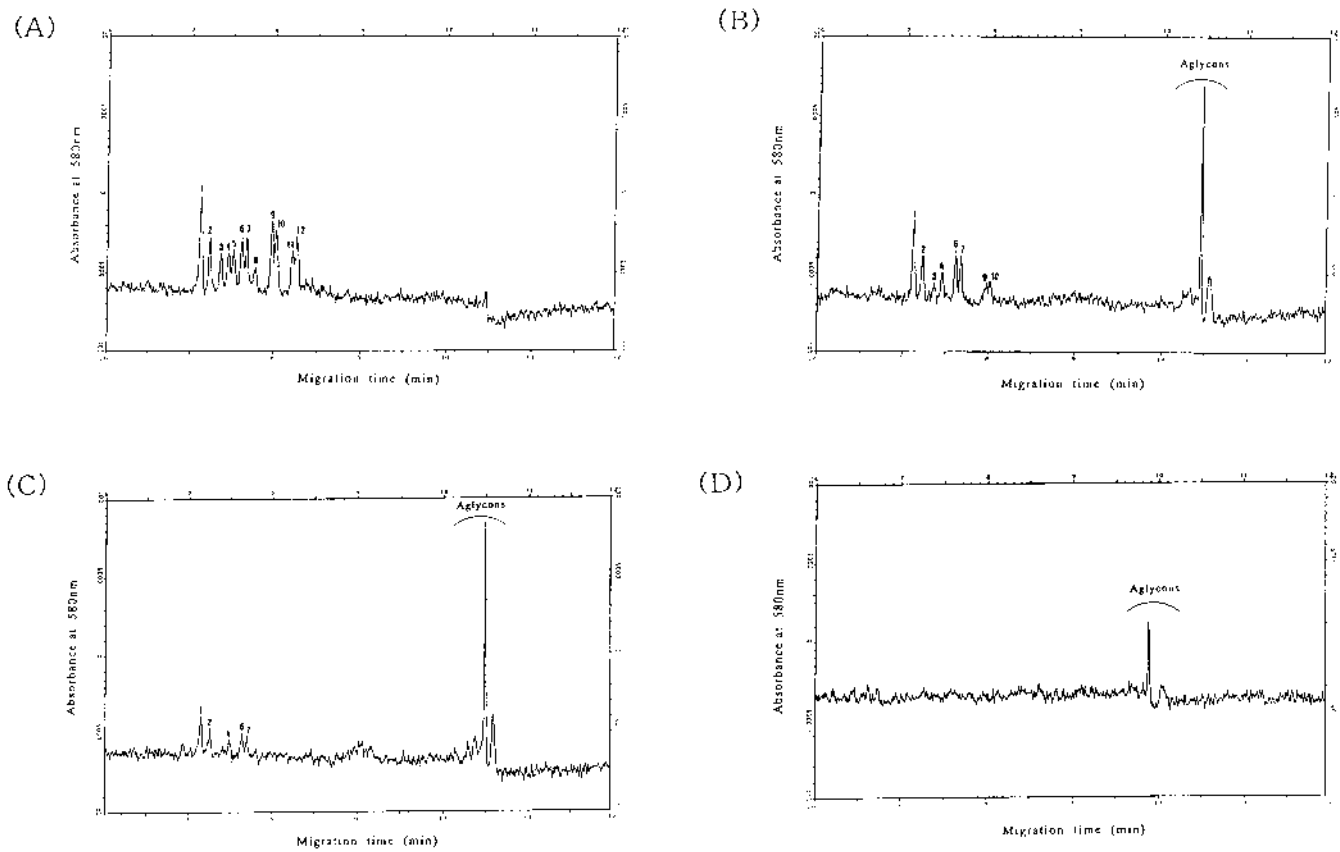


Fig. 2. Change of Anthocyanin Peaks in the CZE Electrophoretogram after TFA Mediated Hydrolysis Reaction

(A) Before hydrolysis reaction, (B) 30 min reactions, (C) 60 min reactions, (D) 90 min reactions. 1, malvidin 3-O-β-D-glucoside; 2, peonidin 3-O-β-D-glucoside; 3, malvidin 3-O-β-D-galactoside; 4, petunidin 3-O-β-D-glucoside; 5, petunidin 3-O-β-D-galactoside; 6, cyanidin 3-O-β-D-glucoside; 7, delphinidin 3-O-β-D-glucoside; 8, unknown 1; 9, cyanidin 3-O-β-D-galactoside; 10, delphinidin 3-O-β-D-galactoside; 11, cyanidin 3-O-β-D-arabinoside; 12, unknown 2.

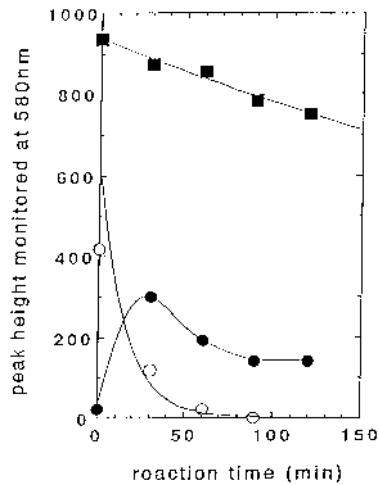


Fig. 3. Time Course Changes of Anthocyanin Disappearance, Aglycon Formation and Bleaching of  $A_{525nm}$  at 95 °C in 9% TFA

a) Bleaching of  $A_{525nm}$  of the reaction solution determined by visible absorption spectroscopy. b) Anthocyanin disappearance and aglycon formation determined by CZE. Symbols: ■,  $A_{525nm}$ ; ○, anthocyanin; ●, aglycon.

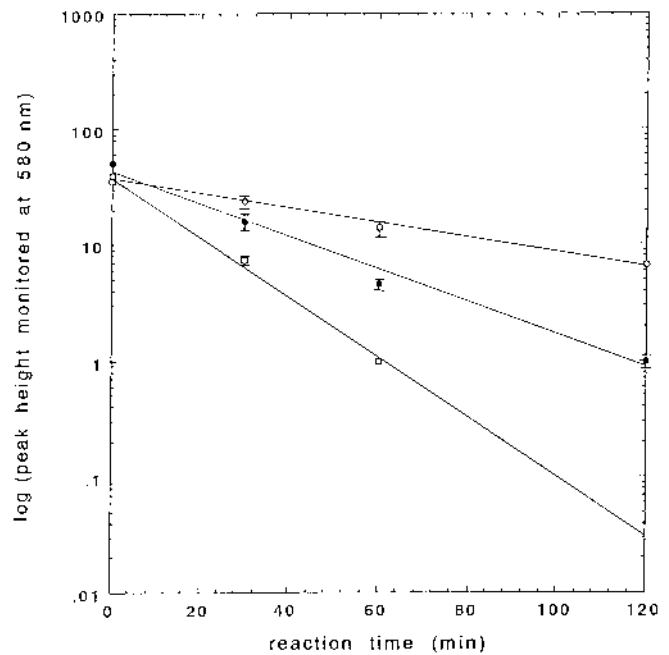


Fig. 4. Effect of Conjugated Sugar on the Rate of Anthocyanin Hydrolysis  
Values are mean ± S.D. of three independent reactions. Symbols: ○, cyanidin 3-O-β-glucoside; ●, cyanidin 3-O-β-galactoside; □, cyanidin 3-O-β-arabinoside.

showed biphasic kinetics, that is, the aglycon peak decreased after the formation reached the maximum after prolonged reaction period. The higher the TFA concentration was, the shorter the time reaching the maximum became (data not shown).

When the changes of CZE peak of each anthocyanin were

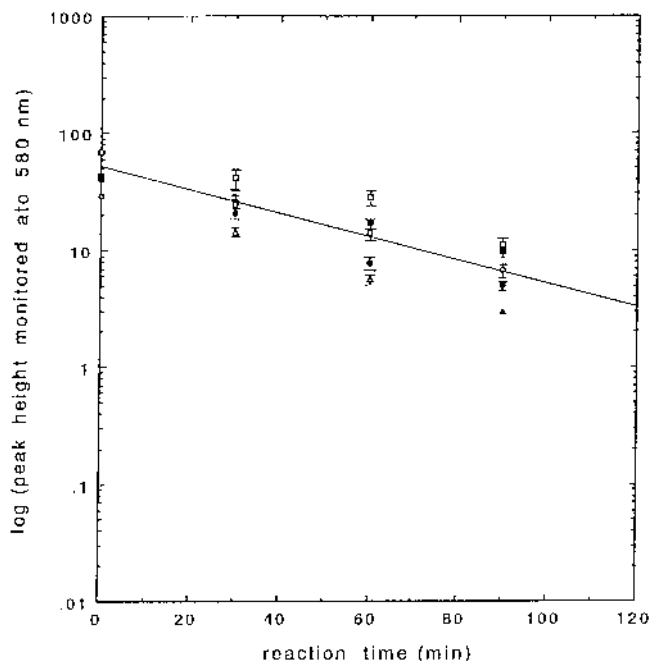


Fig. 5. Effect of Aglycon Structure on the Rate of Anthocyanin Hydrolysis

Values are mean  $\pm$  S.D. of three independent reactions. Symbols:  $\square$ , malvidin 3-*O*- $\beta$ -glucoside;  $\blacksquare$ , peonidin 3-*O*- $\beta$ -glucoside;  $\triangle$ , petunidin 3-*O*- $\beta$ -glucoside;  $\circ$ , cyanidin 3-*O*- $\beta$ -glucoside;  $\bullet$ , delphinidin 3-*O*- $\beta$ -glucoside.

Table 1. Rate Constants of Anthocyanin Hydrolysis in TFA Solution at 95 °C

Anthocyanin	0.1% TFA	1% TFA	3% TFA	9% TFA
Mv 3-Glc	7.6	9.3	21.5	47.2
Mv 3-Gal	17.9	26.2	27.0	52.5
Pn 3-Glc	6.7	6.9	18.5	42.4
Pt 3-Glc	11.4	18.7	27.8	53.4
Pt 3-Gal	21.7	25.9	37.3	37.2
Cy 3-Glc	5.2	12.5	28.9	38.7
Cy 3-Gal	12.2	21.9	43.5	64.3
Cy 3-Ara	26.6	33.7	59.4	117.9
Dp 3-Glc	11.3	15.0	29.4	41.9
Dp 3-Gal	25.6	30.5	42.2	61.1
Unknown 1	21.6	26.8	33.2	43.6
Unknown 2	30.2	34.8	62.7	122.7

First order rate constants of anthocyanin degradation at different TFA concentration ( $\text{min}^{-1} \times 10^{-3}$ ).

plotted against the reaction period, the disappearance rate of the anthocyanin peak was first order (Fig. 4). The kinetic constants thus obtained increased with increasing TFA concentration. When the kinetic constants for the anthocyanins were compared, it was revealed that the anthocyanins having the same aglycon structure showed different kinetic constants depending on their conjugated sugar type. On the other hand, the anthocyanins having the same conjugated sugar showed similar kinetic constants without regard to their aglycon structure (Figs. 4 and 5). Hence the hydrolysis rate of anthocyanins does not depend on the aglycon structure, but on the type of conjugated sugar. The hydrolysis rate constant was found in the following order, arabinoside > galactoside > glucoside in all anthocyanins. The hydrolysis rate constants of each anthocyanin are summarized in Table 1.

The TFA concentration dependence of both the hydrolysis

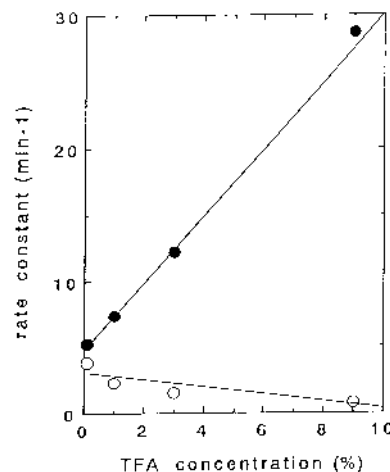


Fig. 6. TFA Concentration Dependence of Anthocyanin Hydrolysis and Bleaching of  $A_{525\text{nm}}$

Symbols:  $\circ$ , bleaching at  $A_{525\text{nm}}$ ;  $\bullet$ , anthocyanin glycoside.

of anthocyanin glycoside and the bleaching of  $A_{525\text{nm}}$  (degradation of anthocyanidin nucleus) are shown in Fig. 6. The hydrolysis rate constant of anthocyanin increased linearly with TFA concentration whereas the rate constant for  $A_{525\text{nm}}$  bleaching decreased with TFA concentration.

## Discussion

The present experiments revealed that Anthocyanin hydrolysis in TFA solution is determined primarily by the conjugated sugar type and not by the aglycon structure. The hydrolysis rate constants for the glycosides were in the following order, arabinoside > galactoside > glucoside without regard to the aglycon structure. Bemisser studied the acid-catalyzed hydrolysis properties of model glycosides of small molecules such as ethanol and benzyl alcohol, and reported similar differences in the hydrolytic behavior of the glycosides such that galactoside was more unstable than glucoside when the glycosides with the same aglycon were compared.<sup>10</sup> Thus, he concluded that the difference in the conformational strain in the sugar moiety was the cause of the kinetic difference of the glycoside hydrolysis. However, his data also showed that the aglycon structure significantly affected the hydrolysis behavior of the glycosides as much as the conjugated sugar. Therefore, it is not yet conclusive that the same mechanism is involved in the anthocyanin hydrolysis, but it is quite interesting that the degradation of anthocyanin is governed primarily by the conjugated sugar structure rather than the aglycon structure.

The present study also identified an unknown anthocyanin (peak 12) from our previous CZE study. We have previously proposed the aglycon structure of peak 12 to be delphinidin from its electrophoretic mobility behavior.<sup>9</sup> In the present study, the degradation rate constant of peak 12 was identical with those for arabinoside, thus it is concluded that the unidentified peak 12 is delphinidin 3-*O*- $\beta$ -D-arabinoside.

From the comparison of the kinetic profiles of anthocyanin disappearance, the aglycon formation, and the bleaching of  $A_{525\text{nm}}$  at different TFA concentrations, the degradation path of anthocyanin was suggested to be different at different pH conditions. At low TFA concentration, the bleaching of  $A_{525\text{nm}}$  of the reaction solution occurred more markedly than

at high TFA and the rate constant was comparable to those of the glycoside hydrolysis and aglycon formation (see Fig. 6). At TFA concentrations higher than 3%, however, the  $A_{525\text{nm}}$  bleaching rate decreased and inversely the glycoside hydrolysis and the aglycon formation were accelerated, thus the time to reach the maximum aglycon formation was shortened. These results indicate that at a low concentration of TFA, the direct bleaching of the anthocyanidin nucleus due to decomposition predominated because of the unstable nature of the flavilium cation. At high TFA concentration, however, the hydrolysis reaction became marked to release the aglycon being transformed successively into another product, probably, the polymerized product. Indeed, a red shift in the  $\lambda_{\text{max}}$  was observed for the product compared to the original anthocyanin solution ( $\lambda_{\text{max}}$  555 nm and 505 nm, respectively, in acidic methanol). Since the secondary product shift absorbs 525 nm light significantly, the observed rate of  $A_{525\text{nm}}$  bleaching could be decreased under the conditions that the reactions (hydrolysis and chromophore decomposition) compete with each other, that is, at high TFA concentration as shown in Fig. 6.

Although the secondary product(s) was suggested to be formed in the reaction mixture as discussed above, we could not detect any additional peak generation in the electrophoretogram. This is because the product was insoluble to the reaction solution since we observed a reddish precipitate at the bottom of the reaction ampules whenever the aglycon peak disappeared in the electrophoretogram. This was further confirmed by the TLC separation of the reactants dissolved in 5% TFA-methanol solution, showing the time dependent increase of the third component concomitantly with the decrease of both anthocyanin and the aglycon spots (data not shown).

Recently, it was reported that polyphenols such as procyanidin, condensed products of anthocyanidin and catechin, is formed in red wine during aging and they have strong antioxidant activity.<sup>11)</sup> If we take account of the solubility and color properties of the new product, in addition to the TLC separation pattern indicating the product has a higher molecular weight than the aglycon, we may suggest that the same type of anthocyanidin polymer was formed in the acidic hydrolysate mentioned above. Further studies are under way to clarify the structure and the antioxidant property of the reddish precipitate.

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