Antioxidative Activity of Monoacylated Anthocyanins Isolated from Muscat Bailey A Grape

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The antioxidative activity of four anthocyanins isolated from the Muscat Bailey A grape, which has a malvidin nucleus, was evaluated according to the amount of malonaldehyde formed by the autoxidation of linoleic acid in Trizma buffer (pH 7.4). Thiobarbituric acid derivatives of malonaldehyde were quantitatively analyzed by a spectrophotometer (at 535 nm). Malonaldehyde (20 nmol/mg of linoleic acid) was regularly detected after a 16-h inculation period without antioxidants. Malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside showed only 2.6 nmol/mg of linoleic acid malonaldehyde production. *p*-Coumaric acid attached to malvidin 3-glucoside and malvidin 3,5-diglucoside was found to play an important role in preventing malonaldehyde production from linoleic acid. The antioxidative activity of an acylated anthocyanin was compared with other chemicals. Malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside proved to be 2 times more effective than commercially available antioxidants such as (+)-catechin and α-tocopherol. From the results, it was found that the monoacylated anthocyanins from the pericarps of grapes can be used as powerful antioxidants as well as for colorants.

Keywords: *Anthocyanin; antioxidants; Vitis sp.; Muscat Bailey A*

The coloring of flowers and fruits, from red through purple to blue, is mostly from anthocyanins, a type of flavonoid. Anthocyanins having multiacylated cinnamic acid derivatives were found to be more stable in a neutral or weakly acidic aqueous solution (Goto et al., 1982; Saito et al., 1971). The complicated chemical structures of anthocyanins have been clarified by using ^{1}H-NMR techniques and mass spectral analysis (Goto et al., 1982, 1984; Saito et al., 1983; Tamura et al., 1983).

Anthocyanins have only recently begun to be regarded as biologically active substances, as well as colorants. For example, anti-inflammatory activities (Vlaskovska et al., 1990), redox potentials (Gabor, 1988), anticonvulsant activity (Drenska et al., 1989), and antioxidative activity (Costantion et al., 1992; Meunier et al., 1989; Igarashi et al., 1989) have been studied. Recently, the antioxidative activity of human low-density lipoprotein caused by the phenolic substances in red wine (at pH 7.4) was also reported by Frankel et al. (1993). Anthocyanins are now being isolated from the residue of pressed grapes used in red wine manufacturing to make colorant for foods and pharmaceuticals (Furtsov et al., 1989).

The precise composition of acylated anthocyanins in grapes determined by using the chemical degradation method, comparison with authentic compounds, and NMR measurements was reported by Williams et al. (1978) and Tamura et al. (1994). Pericarp pigments in Muscat Bailey A were constituted mainly by malvidin derivatives. The grape contains a fairly large amount of acylated malvidin glucosides (in fresh pericarps around 40–50% against total anthocyanin contents).

In this paper, the antioxidative activity of four malvidin derivatives (including malvidin 3-glucoside and malvidin 3,5-diglucoside acylated with *p*-coumaric acid) in the Muscat Bailey A grape was evaluated according to the amount of thiobarbituric acid (TBA) reactive substances (malonaldehyde) formed from oxidized linoleic acid. The activity of those anthocyanins was then compared with that of other antioxidants.

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EXPERIMENTAL PROCEDURES

Analytical Instruments. In the TBA tests, visible absorption spectra were determined using a Hitachi Model 200-15A spectrophotometer (Hitachi, Ltd., Tokyo, Japan) for TBA tests. For the quantitative analysis of anthocyanins, reversed-phase high-performance liquid chromatography (HPLC) was done with a Jasco PU-980 gradient system with a Jasco 875 UV (Jasco Corp., Tokyo, Japan).

(*1*) *Analytical HPLC.* HPLC analysis was carried out according to the method described by Goto et al. (1982) as follows: Column, C_{18}, 5 μm Develosil (250 × 4.6 mm i.d.); temperature, controlled at 40 °C; detector, 280 and 520 nm; mobile phase A, AcOH/CH_3CN/H_2O/H_3PO_4 8:10:80.5:1.5%; mobile phase B, AcOH/CH_3CN/H_2O/H_3PO_4 20:25:53.5:1.5% (v/v); flow rate of the eluent, 1 mL/min. The analysis was accomplished by a linear gradient elution from solvent A to solvent B in 30 min. The typical profile of grape anthocyanins is shown in Figure 1.

(*2*) *Preparative HPLC Conditions for the Isolation of Anthocyanins.* Anthocyanins were isolated using a Jasco SP-45 PG 350D pump equipped with Develosil (Nomura Co., Aichi, Japan): 30 μm ODS glass column (360 × 24 mm i.d.); 10–20 μm ODS stainless column (300 × 10 mm i.d.); and 5 μm ODS stainless column (250 × 20 mm i.d.); temperature, ambient; mobile phase C, AcOH/CH_3CN/H_2O/TFA 20:25:54.5:0.5 (this solvent system was expressed as 100% C). A concentration of solvent was prepared by diluting 100% C with 0.5% TFA aqueous solution. The flow rate of the eluent for the separation was about 4 mL/min.

Isolation of Anthocyanin Pigments. Crude pigments (1.8 g) were extracted from 464 g of Muscat Bailey A pericarps using 1 L of 1% TFA/50% MeOH aqueous solution. The solution was then partially concentrated to remove methanol. The aqueous solution was applied on the Amberlite XAD 7 column (24 × 5.5 cm i.d.) with 0.5% TFA aqueous solution (1 L), and then the anthocyanin fraction was quickly eluted with 0.5% TFA/80% MeOH aqueous solution (1.5 L). The fraction was evaporated under vacuum in dryness. To the residue was added 80 mL of 1% HCl/MeOH and 240 mL of diethyl ether to

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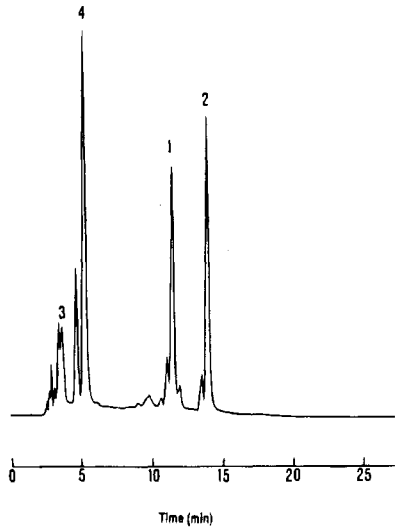

Figure 1. HPLC analysis of pigments in the pericarps of Muscat Bailey A. Peaks: (1) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside; (2) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside); (3), malvidin 3,5-diglucoside; (4) malvidin 3-glucoside. Conditions: column; ODS Develosil 5 μm, 250 × 4.6 mm i.d.; solvent, $CH_3COOH/CH_3CN/H_2O/H_3PO_4$ 8:10:80.5:1.5 (A), 20:25:53.5:1.5 (B); gradient elution from A to B for 30 min; detector, 520 nm.

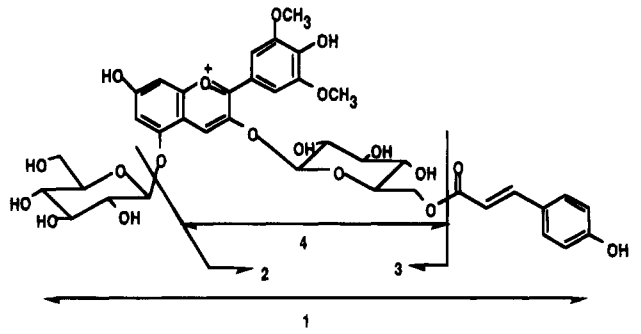

Figure 2. Structure of major anthocyanins isolated from Muscat Bailey A: (**1**) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside; (**2**) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside); (**3**) malvidin 3,5-diglucoside; (**4**), malvidin 3-glucoside.

obtain the precipitate of the pigments. The precipitates were collected by centrifugation (2500 rpm) and then dried in a vacuum desiccator.

The crude pigment was adsorbed on the ODS glass column mentioned above and then fractionated to 11 fractions by using 40% solvent C (fractions 1–6), 60% solvent C (fractions (7 and 8), 80% solvent C (fractions 9 and 10), and 100% solvent C for fraction 11. Purification of pigments was made on a Develosil ODS 10–20 (300 × 10 mm i.d.) or 5-μm (250 × 20 mm i.d.) stainless column. Consequently, fraction 6 (166 mg) gave 63.1 mg of malvidin 3-glucoside, fraction 10 (775 mg) gave 31.9 mg of malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside, and fraction 11 (487 mg) gave 28.9 mg of malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside). The purity of each anthocyanin was checked by analytical HPLC at 290 and 520 nm. Malvin (malvidin 3,5-diglucoside) was purchased from Aldrich Chemical Co. and used without further purification. The four pigments with purity greater than 95% were used to assay antioxidative activity. The chemical structure of the four anthocyanins occurring in the skin of Muscat Bailey A was determined by ^1H NMR and the FRIT-FABMS technique (Tamura et al., 1994) as shown in Figure 2.

Reagents. Ferrous sulfate, butylated hydroxytoluene (BHT), linoleic acid, and *p*-coumaric acid were purchased from Wako Pure Chemicals (Osaka, Japan). (±)-α-Tocopherol, (+)-catechin, and naringenin (flavanone) were purchased from Sigma Chemical Co. (St. Louis, MO). These compounds were used without further purification. Pure water filtered by the Millipore filter produced by Barnstead Co. was used for all steps in this experiment (>15 MΩ·cm).

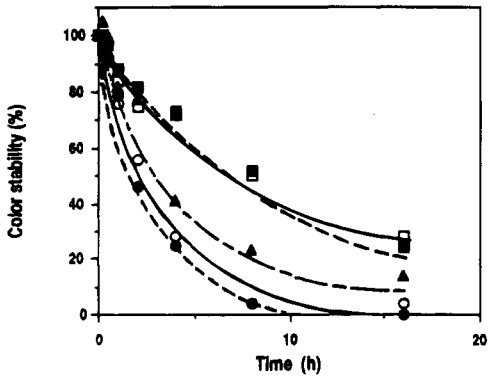

Figure 3. Stability of anthocyanins isolated from Muscat Bailey A in 0.05 M Trizma buffer at pH 7.4 (concentration 10 μM; observed at 540 nm): (□) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside (**1**); (○) malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside) (**2**); (■) malvidin 3,5-diglucoside (**3**); (●) malvidin 3-glucoside (**4**); (▲) crude grape pigments.

Oxidation System of Linoleic Acid by Ferrous Sulfate. The oxidation of linoleic acid was conducted using a modification of a method reported previously (Tamura and Shibamoto, 1991). Linoleic acid (17.8 μmol) was poured into a 30-mL test tube and then diluted with a 4.85 mL of Trizma-buffer solution (0.25 mM, pH 7.4) containing 0.2% SDS (w/v) and 0.75 mM potassium chloride. Trizma buffer was prepared by diluting 6.057 g of tris(hydroxymethyl)aminomethane and 11.184 g of potassium chloride with pure water filter by a Milli pore filter to 1 L after adjusting the pH of the solution to 7.4. Lipid peroxidation was initiated by adding 20 mM ferrous sulfate (0.05 mL). The total volume of the reacting solution was adjusted to 5 mL. Incubation was continued for 16 h at 37 °C in a dark place. The reaction was stopped by putting 90.8 mM BHT alcoholic solution (0.1 mL) in the tube. Each tube contained 2 mg of BHT. The obtained reacted solution (0.2 mL) was used for TBA assay.

Antioxidative Activity Assay. Antioxidant (5 μmol) was diluted with 0.5% trifluoroacetic acid/ethanol, and then the solution was filled to 5 mL. The solution (0.1 mL) containing antioxidants was mixed with the solution (4.9 mL) mentioned above when necessary.

TBA Assay. The antioxidative activity was determined by using the thiobarbituric acid (TBA) method described by Ohkawa et al. (1979). The reacted solution (0.2 mL) mentioned above was derivatized to thiobarbituric acid reactive substances by incubation with 0.67% (w/v) thiobarbituric acid (1.0 mL) and 0.05 N HCl (3.0 mL) for 30 min in a 95 °C water bath. The solution was then cooled in ice for 5 min. The colored substances were extracted by 4.0 mL of 1-butanol. The absorbance of the 1-butanol layer was measured at 535 nm. The results are expressed in terms of malonaldehyde production; a calibration curve was constructed by using malonaldehyde bis(diethyl acetal) (1,1,3,3-tetraethoxypropane) as a standard. Malonaldehyde quantitatively prepared by heat-assisted acid hydrolysis (mentioned above) from an aliquot of 1,1,3,3-tetraethoxypropane (10 mmol/mL) was immediately derivatized to a thiobarbituric acid reactive substance to make the standard calibration curve.

RESULTS AND DISCUSSION

Stability of Anthocyanins. The color stability of malvidin 3,5-diglucoside and malvidin 3-glucoside and their acyl derivatives was measured at pH 7.4 and 37 °C for 16 h and then compared as shown in Figure 3. The decrease in visible absorption (540 nm) of the anthocyanin solution was recorded *vs* time. The color principle in anthrocyanin is due to the chemical structure of the flavylium form or anhydrobase form. During incubation in a weak alkaline aqueous solution, the chemical form of these anthocyanins partially changes to a colorless form such as pseudobase or chalcone (Brouillard and Delaporte, 1977). In a solution of

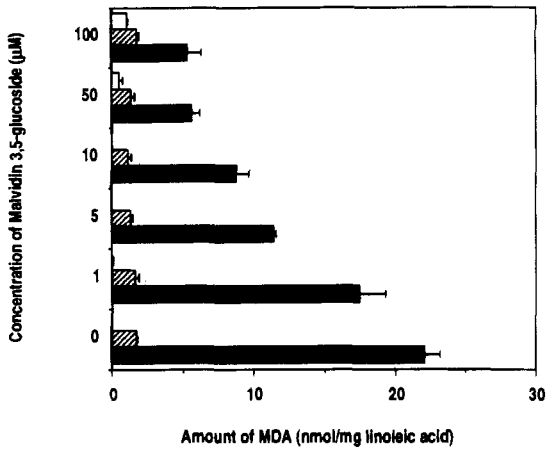

Figure 4. Effect of malvidin 3,5-diglucoside (**3**) on the production of MDA from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.05 M Trizma buffer (pH 7.4): (□) malvidin 3,5-diglucoside (**3**) plus $FeSO_4$; (▨) linoleic acid plus **3**; (■) linoleic acid plus **3** plus $FeSO_4$. Values are mean ± standard deviation ($n = 3$).

malvidin 3,5-diglucoside and malvidin 3-*O*-(6-*O*-coumaroylglucosido)-5-glucoside, color was retained at a level of about 24%. However, in the malvidin 3-glucoside and malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside) solution, the color was almost completely lost. The amount of crude pigment corresponding to 10 μM malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside was adjusted by photospectrometer (520 nm absorbance in 1% HCl/MeOH solution). Crude pigments in grape skin showed intermediate stability between that of malvidin 3-glucoside and that of malvidin 3,5-diglucoside. Antioxidative activity of these anthocyanins was evaluated as the mixture of the chemical forms of each anthocyanin mentioned above.

Anthocyanin Concentration as an Antioxidant. The antioxidative activity was determined by using the thiobarbituric acid (TBA) method. The thiobarbituric acid reactive substances after 16 h of incubation were converted to colored derivatives by incubating those with 0.67% (w/v) thiobarbituric acid and 0.05 N HCl for 30 min in a 95 °C water bath. The absorbance of the colorants was measured at 535 nm. The results are expressed in terms of malonaldehyde production.

It is well-known that anthocyanins with vininal diol on the B ring form a chelate complex with metal (Tamura et al., 1986; Kondo et al., 1992; Shibata et al., 1919). The malvidin skeleton does not have the diol on the B ring, so ferrous sulfate should not be trapped by these anthocyanins and should act only as a reagent to promote autoxidation in an aqueous solution. After 16 h of incubation, using the TBA method, the maximum and minimum levels of lipid peroxidation were determined with or without ferrous sulfate from the amount of malonaldehyde (MDA). As shown in Figure 4, the level of antioxidative activity of malvidin 3,5-diglucoside was dependent on the concentration. Additionally, without linoleic acid, absorbance was not observed at 535 nm using the TBA method when the concentration of malvidin 3,5-diglucoside was less than 50 μM. This means that the TBA method can be used for the estimation of the amount of MDA for anthocyanin concentrations below 50 μM because the absorbance of anthocyanin itself (around 520–540 nm in an acidic solution) does not interfere with that of TBA reactive substances. So, all further experiments were done at a level of less than 50 μM anthocyanin concentration. At 10 μM, malvidin 3,5-diglucoside produced only 8.8 nmol of MDA/mg of linoleic acid content.

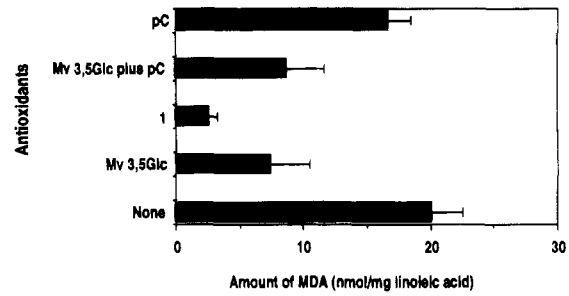

Figure 5. Effect of malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside (**1**) on the production of MDA from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.05 M Trizma buffer (pH 7.4). Concentration of all antioxidants was 10 μM. Values are mean ± standard deviation ($n = 5$). Abbreviations: Mv3,5Glc, malvidin 3,5-diglucoside; pC, *p*-coumaric acid.

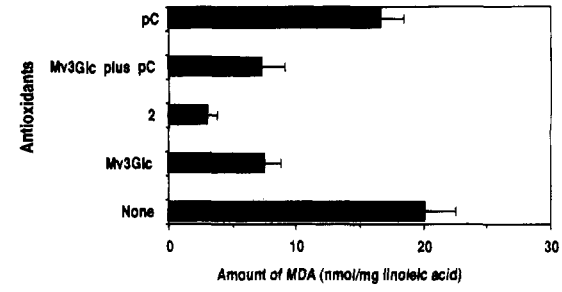

Figure 6. Effect of malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside) (**2**) on the production of MDA from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.05 M Trizma buffer (pH 7.4). Concentration of all antioxidants was 10 μM. Values are mean ± standard deviation ($n = 5$). Abbreviations: Mv3Glc, malvidin 3-glucoside; pC, *p*-coumaric acid.

Effect of Acyl Group. As shown in Figures 5 and 6, the antioxidative activity of a series of malvidin 3,5-diglucoside, malvidin 3-glucoside, and malvidin 3-glucoside and 3,5-diglucoside acylated with *p*-coumaric acid on position 6 of the sugar moiety was evaluated. *p*-Coumaric acid (10 μM) added to an equal amount of malvidin 3,5-diglucoside (nonacylated anthocyanin) did not positively affect the antioxidative activity of malvidin 3,5-diglucoside as shown in Figure 5. However, malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside inhibited the lipid peroxidation of linoleic acid, strongly. This means an intramolecular acyl group (*p*-coumaric acid) enhanced the antioxidative activity of the anthocyanin. This effect was also observed for the malvidin 3-glucoside series (Figure 6), which were unstable in the weak alkaline solution. There was no correlation between color stability and antioxidative activity of the four anthocyanins. Karrer et al. (1927) reported that malvidin 3,5-diglucoside could be converted to a colorless compound (malvone) through oxidation with hydrogen peroxide. The anthocyanin colorless forms (pseudobase of chalcone) or malvone-like compound found at pH 7.4 may show strong antioxidative activity as may the anhydrobase form.

Furthermore, antioxidative activity of aromatic compounds is often observed in those with vicinal methoxyhydroxy or dihydroxy groups. *p*-Coumaric acid, having only a hydroxy group on the aromatic ring, was a weak antioxidant. *p*-Coumaric acid attached to the sugar moiety of the anthocyanin may act as an intramolecule synergist.

Comparison of Antioxidative Activity of Anthocyanins and Other Antioxidants. At a concentration of 10 μM, malvidin 3-(*p*-coumaroylglucosido)-5-glucoside proved to be very effective as an antioxidant, as shown in Figure 7. (+)-Catechin commonly found in tea leaves had a relatively small effect on antioxidative activity.

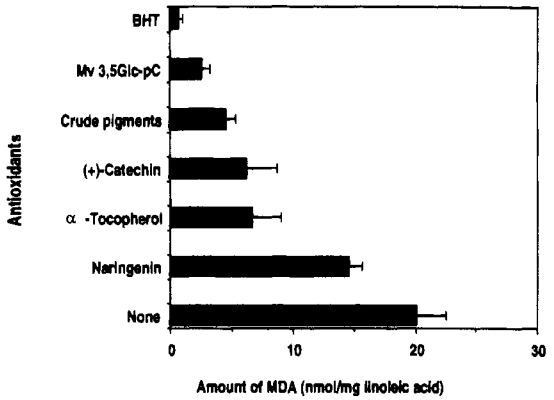

Figure 7. Effect of antioxidants on the production of MDA from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.05 M Trizma buffer (pH 7.4). Concentration of all antioxidants was 10 μM. Values are mean ± standard deviation (n = 5). Abbreviations: Mv3,5Glc-pC, malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside (1).

Naringenin, a precursor of anthocyanin in terms of the biosynthetic pathway, had only a slight effect on oxidation. Antioxidative activity of α-tocopherol and related phenolic antioxidants has been already reported in a self-initiated autoxidation system (Burton and Ingold, 1981). These authors showed α-tocopherol was the strongest antioxidant in the related phenolic compounds. However, in this experiment, α-tocopherol had only a rather modest antioxidant activity. Crude pigments in grape skin (mixture of acylated anthocyanins and nonacylated anthocyanins) also had a slightly larger effect than (+)-catechin, naringenin, and α-tocopherol.

Processed food materials containing anthocyanins such as wine, jelly, and jam are usually preserved under acidic or weakly acidic conditions. However, the results of our research lead us to believe that the anthocyanins in the pericarps of grapes, especially monoacylated anthocyanins, once digested, might act as antioxidants in the human body under the physiological condition of pH 7.4.

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Registry No. Supplied by the Author: (+)-Catechin, 154-23-4; MDA, 542-78-9; Fe, 7439-89-6; linoleic acid, 60-33-3; malvidin 3,5-diglucoside, 16727-30-3; malvidin 3-glucoside, 7228-78-6; malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside, 51939-51-6; malvidin 3-*O*-(6-*O*-*p*-coumaroylglucoside), 34693-53-3; naringenin, 67604-48-2; (±)-α-tocopherol, 10191-41-0; *p*-coumaric acid, 501-98-4.

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