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Antioxidative Activity of Acylated Anthocyanin Isolated from Fruit and Vegetables

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Abstract

The antioxidative activity of seven different acylated anthocyanin pigments isolated from grape, sweet potato, eggplant and red cabbage was evaluated by using linoleic acid autoxidation and rat liver microsomal systems. The acylated anthocyanins were isolated and purified by Amberlite XAD-7, ODS and Sephadex LH-20 column chromatography, and preparative HPLC. Most of acylated anthocyanins exhibited antioxidative activity as strong as α-tocoperol, and especially peonidin 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-feruloylglucopyranosyl)-β-D-glucopyranosyl-5-*O*-β-D-glucopyranoside from purple sweet potato showed the strongest activity, comparable to BHA(not significant, $p<0.05$) in the linoleic acid system. Meanwhile, two acylated anthocyanins from the pericarps of grape and eggplant inhibited considerably the MDA formation from rat liver microsomal lipid peroxidation induced by $FeSO_4/H_2O_2$. In particular, malvidin 3-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucopyranoside from grape pericarps showed the strongest antioxidant activity, comparable to α-tocopherol(not significant, $p<0.05$). These results suggest that the acylated anthocyanins from fruits and vegetables can be used as potential dietary antioxidants and natural colorants.

Key words: acylated anthocyanins, antioxidative activity, fruit and vegetables

INTRODUCTION

There is considerable interest in the development of natural food colorants to replace synthetic food colorants. In particular, anthocyanin pigments have considerable potential in the food industry as safe and effective food additives due to apparently harmless to health(1). Compared to the synthetic colorants, however, anthocyanins in foods are susceptible to color deterioration during processing and strage because of their instability towards a variety of chemical and physical factors(2).

There is a continuing effort to find more stable anthocyanins in a neutral or weakly acidic aqueous solution. In particular, the polyacylated anthocyanins displaying marked stability have recently been isolated and characterized from the plants(3-7). It was reported that their high stability was due to acyl groups which could stack above and below the active site, thereby inhibiting the access of water to form the pseudobase(hemiketal)(8).

Recently, anthocyanins have been recognized as biologically active substances, including anticonvulsant(9) antiinflammatory(10), and antioxidative activities(11-13). Tsuda et al.(14,15) suggested that anthocyanins may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems. Athocyanins together with other flavonoids were also found to be important factors in the inhibition of lipid peroxidation of human low-density lipoprotein(16). Thus, much attention has been focused on the antioxidative activity of anthocyanins, and especially the acylated anthocyanins isolated from some foods with high activity have been reported(17-19).

The objective of this study was to evaluate the antioxidative activity of seven different acylated anthocyanins isolated from grape, sweet potato, eggplant, and red cabbage using by linoleic acid autoxidation and rat liver microsomal systems.

MATERIALS AND METHODS

Materials and reagents

The fresh sweet potatoes(*Ipomoea batatas*), grapes (*Vitis vinifera*), eggplants(*Solanum melongena*) and red cabbages(*Brassica oleracea*) were available from the market. Butylated hydroxyanisole(BHA), linoleic acid, and α-tocopherol were purchased from Wako Pure Chemical

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Industries Ltd.(Osaka, Japan). H_2O_2, ferrous sulfate, trifluoroacetic acid(TFA), tetramethylsilane(TMS), CF_3CO_2D, and DMSO-d_6 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in this study were of analytical grades.

Isolation and purification of anthocyanin pigments

Isolation and purification of acylated anthocyanins were performed by the method described by Goto et al.(3) with a slight modification.

Crude anthocyanin pigments(2g) were extracted from the pericarps of grapes, sweet potatoes and eggplants, and the leaves of red cabbages using 0.5% TFA in 30% CH_3CN aqueous solution for overnight. The solution was concentrated to a small volume *in vacuo*, and then applied onto the Amberlite XAD-7(Organo Chemical, Japan) column(5cm i.d.×30cm) with 0.5% TFA aqueous solution. The column was washed stepwise with distilled H_2O, 20% MeOH, and then anthocyanin fraction was eluted with 0.5% TFA in 80% MeOH aqueous solution. The evaporated pigment fraction was dissolved in a small amount of 1% HCl in MeOH, and the anthocyanin was precipitated by the addition of diethyl ether. The precipitates were collected by centrifugation(2,500rpm) and then dried in a vacuum desiccator.

The partially purified pigments were further absorbed on the ODS(40-63μ, Sigma Chemical Co.) glass column (3cm i.d.×20cm), and then fractionated to 4 fractions by using HOAc/CH_3CN/H_2O solvent system. In general, the acylated anthocyanin pigments were eluted with HOAc/CH_3CN/H_2O(20 : 20 : 60, v/v/v) solution. Finally, isolated pigments were purified on a Waters Delta Prep 4000 HPLC(Waters, Milford, MA, USA) using ODS-5 stainless column(10-20μm, 20mm i.d.×25cm, Nomura Chem. Co., Aichi, Japan) at 40°C with flow rate of 5ml/min monitoring at 535nm. Solvent systems used were as follows; a linear gradient elution for 50min from 20 to 90% solvent B (1.5% H_3PO_4, 20% HOAc, 35% CH_3CN in H_2O) in solvent A(1.5% H_3PO_4 in H_2O). Each fraction of these purified pigments was dissolved in a small volume of 1.0% HCl-MeOH, followed by addition of excess Et_2O, and then drying to give seven pigment powers; sweet potato 1(ca 5mg), 2(ca 13mg), and 3(ca 11mg), grape 1(ca 25mg) and 2(ca 30mg), eggplant(ca 34mg), and red cabbage(ca 29mg).

Thin-layer chromatography(TLC) analysis

TLC of seven purified anthocyanins from samples was carried out on cellulose plate(10×10cm, 0.2mm, Merck, Darmstadt, Germany), by using following solvent systems; BAW(n-BuOH/HOAc/H_2O, 4 : 1 : 5, upper phase), BuHCl(n-BuOH/2MHCl, 1 : 1).

Instrumental analysis

(a) UV-visible spectrophotometer : UV-visible aborption spectra of purified anthocyanins were recorded on a spectrophotometer(Spectronic Genesys, Milton Roy, USA) in 0.1% HCl in MeOH.

(b) ^{1}H-NMR and FAB-MS spectrometry: ^{1}H-NMR (500MHz) spectrum was obtained by a AMX-500 spectrometer(Bruker, Germany) in DMSO-d_6-CF_3CO_2D(9 : 1) containing tetramethylsilane(TMS) as an internal standard. The fast atom bombardment mass(FAB-MS) spectrum was recorded on a JEOL JMS-AX505 WA(Japan Electron and Optics Laboratory Co. Ltd., Tokyo, Japan) with glycerol as the mounting matrix.

Antioxidative activity of acylated anthocyanins in the linoleic acid system

Autoxidation of linoleic acid in the water-alcohol system was carried out by using the method of Osawa and Namiki(20). Each sample(100μg) was added to a solution mixture of linoleic acid(0.13ml) and 99.0% distilled ethanol(10ml), with a 50mM phosphate buffer(pH 7.0, 10 ml); the total volume was adjusted to 25ml with distilled water. The solution was mixed in a conical flask and incubated at 40°C. At intervals during incubation, the degree of oxidation was measured by the thiocyanate method (21) by reading the absorbance at 500nm after coloring with $FeCl_2$ and thiocyanate.

Antioxidative activity of acylated anthocyanins in the rat liver microsomal system

SD rats were obtained from Dae Han Laboratory animal research center Co. Ltd.(Eumsung, Korea) and fed commercial diet. After 10 days, liver was immediately removed under anesthetization and microsome was isolated by standard differential centrifugation techniques, and stored at -70°C until needed.

Lipid peroxidation of microsome was induced by $FeSO_4$/H_2O_2 as a hydroxy radical generator(22). Microsome was incubated with 50mM phosphate buffer(pH 7.4), each sample solution(100μg/assay) and 3μM $FeSO_4$/10μM H_2O_2 for 20min, and TBARS produced were determined according to TBA assay reported by Ohkawa et al.(23). The inhibi-

tion rate of lipid peroxidation of each sample was calculated as follows; {1-(A_{535} of reaction mixture with sample/ A_{535} of control)}×100.

Statistical analysis

Statistical analysis was accomplished with Statistical Analysis System(24) software package on replicated test data. Significant differences between means were determined by Duncan's Multiple Range test($p<0.05$).

RESULTS AND DISCUSSION

Isolation and purification of acylated anthocyanins

Seven different acylated anthocyanins from four samples were isolated and purified according to the method described previously. The chromatographic and spectral data of purified seven acylated anthocyanins are shown in Table 1. In the UV-VIS spectra, the presence of the characteristic absorption at the range of 300~330nm suggested that all pigments were acylated anthocyanin with aromatic acids (5,6). In addition, the ratio of E_{acyl}/E_{max} of all anthocyanins was higher than that of simple anthocyanin glycosides, indicating that the presence of one or two acylated anthocyanins(7).

Antioxidative activity of acylated anthocyanins

Antioxidative activities of seven different acylated anthocyanins as measured by the thiocyanate method in the linoleic acid system are shown in Fig. 1. Most of acylated anthocyanins showed antioxidative activity as strong as the standard antioxidant, α-tocopherol. Especially, the acylated anthocyanins 2 isolated from the sweet potatoes exhibited the strongest antioxidative activity, comparable to BHA(not significant, $p<0.05$). In the previous report (25), we identified anthocyanin 2 from purple sweet potato as 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-feruloylglucopyranosyl)-β-D-glucopyranosyl-5-*O*-(β-D-glucopyranosyl)-peonidin by using ^{1}H-NMR and FAB-MS spectroscopy. This result supports an earlier report of the high antioxidative properties of acylated anthocyanins with several sugar moieties, as compared to non-acylated and deacylated anthocyanins(12,18,19).

Meanwhile, in the $FeSO_4/H_2O_2$-dependent lipid peroxidation system using a rat liver microsome(Fig. 2), acylated anthocyanin **2** from the pericarps of grapes showed the strongest antioxidative activity, comparable to α-tocopherol(not significant, $p<0.05$). Acylated anthocyanins

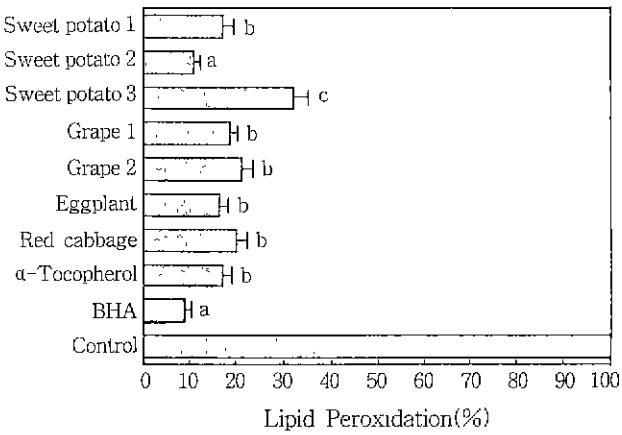

Fig. 1. Antioxidative activity of seven different acylated anthocyanins isolated from fruit and vegetables in the linoleic acid system.
The activity was determined by the thiocyanate method. Reportes values are the mean±SD(n=3). Values in each column with similar letters are not siginificantly different($p<0.05$). A control without an added samples or standard antioxidants represents 100% lipid peroxidation

Table 1. R_f values, chromatographic and spectral properties of seven different acylated anthocyanins isolated from fruit and vegetables

Anthocyanin	R_f values[1] (×100) BAW	BuHCl	λ_{max}(nm)	Spectral data[2] E_{acyl}/E_{max}(%)	$AlCl_3$	R_t[3] (min)
Sweet potato 1	30	15	529,296,330	98	−	30.8
Sweet potato 2	40	18	529.295,330	95	−	31.1
Sweet potato 3	43	25	526.285,331	51	−	35.3
Grape 1	47	29	543,285,320	74	−	38.8
Grape 2	54	33	547,284,320	74	−	41.4
Eggplant	26	12	546,280,309	76	+	27.2
Red cabbage	44	27	537,300,333	109	−	37.4

[1]Developed in cellulose plate by descending method at 25℃ using following solvent systems; BAW(n-BuOH/HOAc/H_2O, 6:1:2); BuHCl(n-BuOH/2M HCl, 1:1)
[2]Determined in 0.1% HCl in MeOH
[3]Retention time in HPLC analysis. HPLC condition is the same as the Materials and Methods

from grape and eggplant also exhibited high antioxidative activity, although their activities were weaker than α-tocopherol. Other acylated anthocyanins showed weaker antioxidative activities than three above acylated anthocyanins. From these results, we found that the acylated anthocyanins from grapes had strong antioxidative activity not only in the autoxidation of the linoleic acid system but also in the cell membrane model system like rat liver microsome. These results also support that the anthocyanin colorless forms(pseudobase of chalcone) or malvone-like compound found at pH 7.0 may be mainly responsible for the strong antioxidative activity of acylated anthocyanin of grape(18). However, there is a somewhat difference among the antioxidative activities of seven acylated anthocyanins determined by using above two systems. The difference may be due to experimental conditions, including the solubilities and partitioning of acylated anthocyanins between the aqueous and lipid phases of assay system.

Identification of anthocyanin 2 of grapes

Grape anthocyanin 2 showing strong antioxidative activivity in above two lipid peroxidation system was identified by ^{1}H-NMR and FAB-MS spectroscopy. ^{1}H-NMR spectrum, as measured in DMSO-d_6-TFA-d_1(9 : 1), showed the presence of a malvidin nucleus, *p*-coumaric acid, and glucose(Table 2). Four kinds of aromatic proton at δ8.69, 6.65, 6.91, 7.88 and methoxyl group at δ3.85(6H) indicated this anthocyanin has the malvidin skeleton.

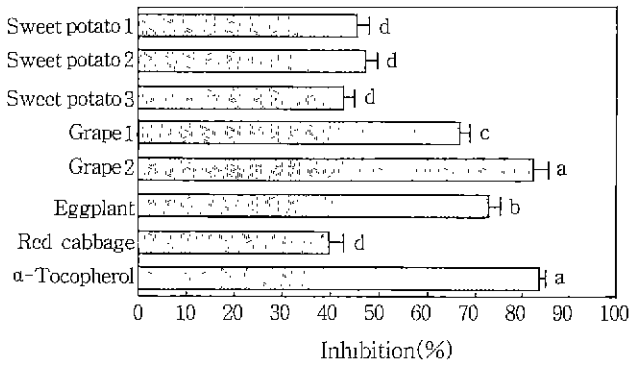

Fig. 2. Inhibition of $FeSO_4/H_2O_2$-dependent lipid peroxidation in the rat liver microsome system by seven different acylated anthocyanins isolated from fruit and vegetables.
The concentration of sample tested was 100μg/assay.
Details are given in Materials and Methods
Reported values are the mean±SD(n=3).
Values in each column with similar letters are not significantly different($p<0.05$).

Table 2. ^{1}H-NMR analysis of malvidin 3-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucopyranoside(MCG) isolated from the pericarps of grapes in DMSO-d_6-CF_3COOD(9:1)

Positoin	MCG
Aglycone	
H-4	8.69s
H-6	6.65d (1.7)
H-8	6.91d (1.7)
H-2'& 6'	7.88s (2H)
H-3'& 5'(OMe)	3.85s (6H)
Anomeric H	5.37d (7.6)
Glu CH_2	4.2~4.6
Glucosyl	3.2~3.9
p-Coumaryl	
H_α	6.16d (15.8)
H_β	7.33d (15.8)
H-2''& 6''	7.26d (2H, 8.8)
H-3''& 5''	6.70d (2H, 8.8)

*J(Hz) in parentheses

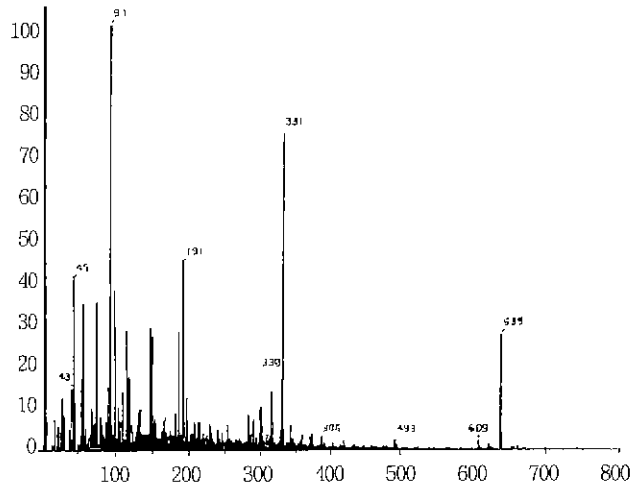

Fig. 3. FAB-MS spectrum of malvidin 3-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucopyranoside isolated from the pericarps of grapes.

Anomeric proton at δ5.37 was assigned as glucose according to their multiplicity and spin-spin coupling constants(7). Glucose must be β-glucopyranoside form based on the vicinal coupling constant with J value of 7.6Hz. Four doublets with coupling constants of 15.8Hz appeared to be the *trans* olefinic protons H-α and H-β. The four aromatic protons(H-3'', 5'' & H-2'', 6'') exhibited as two AX systems, indicating the presence of the 1,4-disubstitued pattern of *p*-coumaric acid. Furthermore, *p*-coumaric acid was determined to be bonded to C-6 hydroxyl group of glucose by the observation of low-field shifts of methylene protons(H-6a & H-6b) of glucose at δ4.27. Meanwhile, the FAB-MS of grape anthocyanin 2 gave its mol-

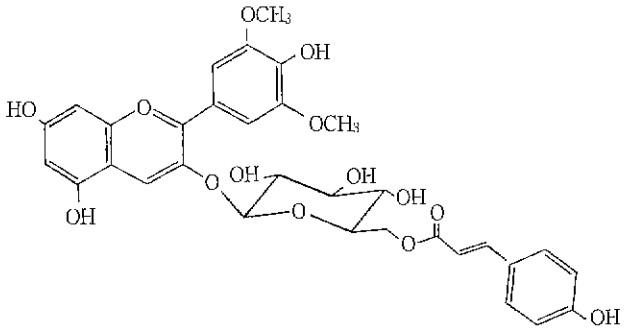

Fig. 4. Chemical structure of malvidin 3-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucopyranoside isolated from the pericarps of grapes.

ecular ion peak [M$^+$] at 639m/z. Two significant fragment ion peaks at m/z 493 [639(M)-146(*p*-coumaric acid unit)] and at m/z 331 [493(M)-162(glucose unit)] were observed in the FAB-MS spectrum(Fig. 3). On the base of these data, grape anthocyanin 2 was determined to be malvidin 3-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucopyranoside(MCG)(Fig. 4). We report for the first time to speculate that MCG can play an important role in the prevention of lipid peroxidation of cell membranes induced by hydroxyl radical, although the structural elucidation and antioxidative property of MCG and its derivatives were already reported (18,26). In addition, this result supports a previous report that the antioxidative activity of acylated anthocyanins is enhanced markedly by an intramolecular acyl group (*p*-coumaric acid) attached to the sugar moiety of the anthocyanins(12,18).

These results suggest that the acylated anthocyanins in several fruits and vegetables can be used as natural food colorants and dietary antioxidants. Especially, it is very interesting to note that anthocyanin pigments ingested through plant-based daily diets can contribute to the prevention of lipid peroxidation, which was strongly associated with atherosclerosis, carcinogenesis and aging (14). Therefore, further investigation on the antioxidative activity of acylated anthocyanin pigments in an *in vivo* system is needed.

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