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CYANIDIN 3-MALONYLGLUCOSIDE AND MALONYL-COENZYME A: ANTHOCYANIDIN MALONYLTRANSFERASE IN *LACTUCA SATIVA* LEAVES

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Key Word Index—*Lactuca sativa*; Compositae; leaves; malonylated anthocyanin; cyanidin 3-*O*-(6″-malonylglucoside); biosynthesis; malonyltransferase.

Abstract—A pigment isolated from *Lactuca sativa* leaves was identified as cyanidin 3-*O*-(6″-malonylglucoside) by chromatographic and spectral methods. The acyltransferase extracted from the leaves catalysed the malonylation of anthocyanidin 3-glucosides with malonyl-CoA as an acyl donor. This enzyme was characterized in more detail.

INTRODUCTION

Although the major anthocyanin in cyanic leaves of *Lactuca sativa* had been previously identified as cyanidin 3-glucoside (Cy 3G) [1], evidence that it was acylated with an aliphatic acid was obtained during plant surveys [2]. Acyl groups in zwitterionic anthocyanins are labile and are frequently lost during extraction procedures [3, 4]. This suggested that the re-examination of anthocyanin in leaves of *L. sativa* would be worthwhile.

A recent survey of the acyltransferase responsible for the aliphatic acylation in flowers of four species in Compositae reveals that the enzyme transfers an acyl group via a respective CoA-ester to the sugar moiety of anthocyanins [5–8]. Every enzyme so far investigated, however, has been isolated only from flowers. We, therefore, were also prompted to examine the acyltransferase extracted from leaves in this plant. This paper deals with identification of the anthocyanin from cyanic leaves of *L. sativa* and characterization of the acyltransferase present.

RESULTS AND DISCUSSION

The major anthocyanin was extracted with 5% formic acid from fresh purple leaves of *L. sativa* cv. Red Fire and purified successively by HP-20 column chromatography, paper chromatography and HPLC. This anthocyanin (**1**) showed nearly the same values in R_f, R_t and UV–visible spectra with those of cyanidin 3-*O*-(6″-malonylglucoside) (Cy 3MG) prepared from *Dendranthema morifolium* [9] and converted into Cy 3G by alkaline deacylation (Table 1), suggesting that **1** was Cy 3G acylated with malonic acid. The IR spectrum for **1** indicated the presence of an acylating aliphatic acid by the ester carbonyl absorption at 1720 cm^{-1}. The deacylation product of **1** was analysed by TLC, which showed, in fact, the presence of a

Table 1. Chromatographic and spectral properties of anthocyanin **1** from *Lactuca sativa* leaves

	$R_f \times 100$ in				R_t	In 0.01% HCl–MeOH		
Anthocyanin	AHW	AW	BAW1	BH	(min)	λ_{max} (nm)	E_{440}/E_{vis}	$AlCl_3$*
Anthocyanin **1**	22	38	42	26	9.3	282, 529	22	+
Deacylated anthocyanin of **1**	16	30	32	14	4.9	282, 529	23	+
Authentic anthocyanins								
Cy 3G	16	30	32	15	4.7	282, 529	23	+
Cy 3MG	22	37	42	26	9.3	282, 529	22	+

*$AlCl_3$ shift was observed after addition of 5% $AlCl_3$–MeOH.

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Table 2. TLC analysis of compounds produced by deacylation and hydrolysis of anthocyanin **1**

Compound	$R_f \times 100$ in BAW2	EAA	EFW	ETN	Colour with BCG*	AHP†
Malonic acid and acylating acid of **1**	67	71	76	7	Yellow	ND‡
Glucose and acid hydrolysate of **1**	30	17	26	42	ND	Brown
H_2O_2 hydrolysate of **1**	32	48	38	32	Yellow	Brown
Related compounds						
Succinic acid	78	82	82	16	Yellow	ND
Malic acid	56	59	62	12	Yellow	ND

*Bromocresol green spray reagent for organic acids.
†Aniline hydrogen phthalate spray reagent for reducing sugars.
‡Not detected.

malonic acid as the acylating acid linked to the glucose (Table 2). The structure of **1** was further confirmed by SI mass spectral measurement, which gave the molecular ion at *m/z* 535. Thus, the anthocyanin **1** is a malonylated Cy 3G, and the previous finding of Cy 3G [1] was due to the loss of the acyl group during pigment extraction with methanolic HCl.

A reaction product was obtained by incubation of a mixture containing crude extracts from cyanic leaves of *L. sativa* cv. Red Fire, Cy 3G and malonyl-CoA as an acyl donor. The product was identified as Cy 3MG (Table 3), which was identical to **1**.

The malonyltransferase showed maximum activity at pH 7.5 with half maxima at pH 6.2 and 8.7. The acylation reaction was saturated in turn with peonidin 3-glucoside (Pn 3G) at 300 μM, the 3-glucosides of cyanidin, pelargonidin (Pg 3G), delphinidin (Dp 3G), petunidin (Pt 3G) and malvidin (Mv 3G) at 500 μM, and with malonyl-CoA at 200 μM. The calculated E_a was 61 and 182 KJ mol^{-1} following denaturation.

None of the divalent metal ions tested had a significant effect on the acylation reaction except for Cu^{2+}, Fe^{2+} and Zn^{2+}, which inhibited it to 28, 34 and 83%, respectively. No significant effect was observed on the enzyme reaction by the inhibitors, diethylpyrocarbonate, diethyldithiocarbamate and *N*-ethylmaleimide.

Crude extracts were also prepared from acyanic leaves of cv. Green Waves and examined for specific activities using as substrates Cy 3G and three CoA-esters (Table 4). The acyanic cultivar showed high activities (*ca* 20% of cyanic cultivar) of malonyltransferase and succinyltransferase, even though the latter's activity was very low. This result suggests that a gene(s) for the acylation of anthocyanins is likely to be expressed independently of the genes for anthocyanidin biosynthesis as found in *Dendranthema morifolium* [6] and *Zinnia elegans* [7].

Typical 3-glucosides and 3,5-diglucosides of anthocyanidins were tested as acyl acceptors by the standard enzyme assay method using enzyme extracts from cv. Red Fire (Table 5). The Cy 3G and Pg 3G are nearly equivalent substrates for the malonyltransferase as found for all aliphatic acyltransferase so far examined [5–8]. In addition, all of the other anthocyanidin 3-glucosides tested, served similarly as acyl acceptors. In contrast, 3,5-diglucosides of cyanidin (Cy 3G5G) and pelargonidin (Pg 3G5G) did not serve at all, as we previously found [6–8]. The aliphatic acyltransferase has a broad substrate specificity for the aglycone in the anthocyanin, but not for the glycoside.

Table 3. Chromatographic properties of anthocyanin produced by the acyltransferase reaction

Compound	$R_f \times 100$ in AHW	BFW	BH	FW	R_t (min)
Reaction product	16	55	18	16	21
Deacylated reaction product	12	46	10	13	15
Authentic anthocyanins					
Cy 3MG	16	55	18	16	21
Cy 3G	12	46	10	13	15

Table 4. Specific activity of acyltransferase in cyanic and acyanic leaves of *Lactuca sativa*

Cultivar	Leaf colour	Content of Cy 3MG (%)	Specific activity ($10^{-1} \times$ pkat μg^{-1}) Malonyl-CoA	Succinyl-CoA*	Acetyl-CoA*
Red Fire	Purple	95	11.92	1.00	0
Green Wave	Green	0	2.49	0.15	0

*The reaction was performed using 600 μM succinyl-CoA or 300 μM acetyl-CoA. The reaction product was analysed using the acylated anthocyanin produced by succinyltransferase [8] or acetyltransferase [7].

Table 5. Substrate specificity of the malonyltransferase for various anthocyanins

Anthocyanin	Relative activity* (%)	Concentration (μM)
Cy 3G	100	500
Pg 3G	92	500
Pn 3G	95	300
Dp 3G	137	500
Pt 3G	86	500
Mv 3G	122	500
Cy 3G5G	0	500
Pg 3G5G	0	500

*The malonyltransferase activity obtained with Cy 3G was expressed as 100%.

EXPERIMENTAL

Plant material. Plant leaves of *L. sativa* cvs Red Fire and Green Wave were collected at the farm of Minami-Kyushu University.

Chemicals. Authentic anthocyanins were isolated by the method of ref. [10] and further purified by prep. HPLC using an Inertsil ODS-2 column (GL Science) and HOAc solvent system. Aliphatic CoA-esters were purchased from Sigma.

Isolation and purification of anthocyanin. Pigments were extracted from leaves with 5% HCO_2H at room temp. for *ca* 12 hr. The extracts were placed on a pre-washed Amberlite HP-20 CC and eluted with 5% HCO_2H–MeOH. The condensed eluate was purified successively by PC (Toyo No. 526) in solvents of BAW1 (*n*-BuOH–HOAc–H_2O) (6:1:2), BFW (*n*-BuOH–HCO_2H–H_2O) (4:1:2), FW (HCO_2H–H_2O) (1:9) and BFW, followed by eluting pigments from the paper with 5% HCO_2H. The condensed eluate was further purified by HPLC as above.

Anthocyanin identification. Purified **1** was analysed by TLC, HPLC and UV–VIS spectrometry, and further confirmed by SIMS and IR. Deacylation with alkali, H_2O_2 oxidation and acid hydrolysis were performed by standard procedures [11].

TLC and HPLC analysis. TLC was performed on microcrystalline cellulose plates (Avicel SF, Funakoshi) in solvents, AHW (HOAc–HCl–H_2O) (15:3:82), AW (HOAc–H_2O) (3:17), BAW1, BAW2 (*n*-BuOH–HOAc–H_2O) (4:1:2), BFW, BH (*n*-BuOH–1 N HCl) (1:1), EAA (EtOAc–HOAc–H_2O) (3:1:1), EFW (Me_2CO–HCO_2H–H_2O) (5:2:1) and ETN (EtOH–NH_4OH–H_2O) (16:1:3). HPLC was carried out by the same methods described previously [6] except for analysis of **1** (Table 1), in which the detection was carried out on an Inertsil ODS-2 column 250 × 4.5 mm i.d., 5 μm) with linear gradient elution from 40 to 80%.

Instrumental analysis. IR spectra were recorded in KBr, and UV spectra in 0.01% HCl–MeOH. SIMS were determined on a Hitachi M-80B mass spectrometer using Xe gas in DMSO with BzOH as matrix.

Cyanidin 3-malonylglucoside. IR ν_{max} cm^{-1}: 3424, 1720 (ester C=O), 1644, 1610; SIMS *m/z*: 535 $[M]^+$ ($C_{24}H_{23}O_{14}^+$ requires 535 as the flavylium ion).

Enzyme peparation, protein determination and enzyme assay. Enzymes were prepd from the leaves essentially by the same methods described previously [6]. Protein content of the prepn was determined by the method of ref. [12]. For the enzyme assay, the standard reaction mixt. contained in a total vol. of 100 μl, 10 μl enzyme extract, 500 μM Cy 3G, 200 μM malonyl-CoA and 70 μl of 0.1 M K-Pi buffer (pH 7.5) containing 10 mM L-cysteine. The reaction mixt. was incubated at 35° for 20 min and stopped by addition of 50 μl $CHCl_3$–MeOH (2:1, 5% HCO_2H) resulting in a Folch partition [13]. Anthocyanins in the upper phase were analysed by HPLC, and also in part by TLC.

Characterization of enzyme. Enzymes in crude extracts were characterized by standard procedures [6], unless otherwise noted. For the substrate specificity test, malonylated forms of Pn 3G, Dp 3G, Pt 3G and Mv 3G were identified by co-chromatography using the authentic anthocyanins purified from flowers of *Hibiscus syriacus* [14].

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