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SUCCINYL-COENZYME A: ANTHOCYANIDIN 3-GLUCOSIDE SUCCINYLTRANSFERASE IN FLOWERS OF *CENTAUREA CYANUS*

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Key Word Index—*Centaurea cyanus*; Compositae; flower buds; anthocyanin biosynthesis; acylated anthocyanins; succinyltransferase.

Abstract—The acyltransferase from blue flowers of *Centaurea cyanus* containing succinylated cyanidin 3,5-diglucosides catalysed the transfer of the succinyl moiety from succinyl-CoA to 3-glucosides of cyanidin and pelargonidin, but not to 3,5-diglucosides. Similar results were obtained using the enzyme from pink flowers containing succinylated pelargonidin 3,5-diglucosides. The enzyme also catalysed these anthocyanidin 3-glucosides to malonylate at nearly the same rate as succinylation.

INTRODUCTION

Until now, aliphatic acylation of anthocyanins has been demonstrated enzymatically with malonyltransferase in flowers of *Callistephus chinensis* [1] and *Dendranthema morifolium* [2], and also with acetyltransferase in those of *Zinnia elegans* [3]. The acyltransferase in these plants catalyses the aliphatic acyl transfer from a respective CoA-ester to the sugar moiety of anthocyanins, leading in an analogous manner to aromatic acylation [4, 5]. In *Zinnia* containing cyanidin 3,5-diglucosides (Cy 3G5G) and pelargonidin 3,5-diglucoside (Pg 3G5G) acylated with acetic acid, evidence that the acylation of anthocyanidin 3-glucoside preceeds 5-*O*-glucosylation has also been obtained by analysis of substrate specificity of the acyltransferase [3]. In addition, the analysis of acyltransferase from flowers of *Dendranthema* [2] and *Zinnia* [3] provides information that a considerable amount of the enzyme is synthesized even in acyanic flowers, implying that a gene for the acyltransferase is expressed independently of anthocyanidin biosynthesis in these plants.

Among aliphatic acylation of anthocyanins, the occurrence of succinylation appears to be rare in nature, and to be restricted to the tribe Cynareae of the Compositae [6, 7]. In *Centaurea cyanus*, blue and pink flowers were found to contain succinylated 3,5-diglucosides of cyanidin (Cy 3SG5G) [8, 9] and pelargonidin (Pg 3SG5G) [10], respectively. The acyltransferase of this plant, however, has not yet been elucidated. We now report the enzymatic properties and distribution of the succinyltransferase in cyanic and acyanic flowers of *C. cyanus*.

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RESULTS AND DISCUSSION

HPLC analysis revealed a new product to be formed in the reaction mixture when crude extracts from blue flowers of *Centaurea cyanus* cv Kanzaki-yae were incubated with cyanidin 3-glucoside (Cy 3G) and succinyl-CoA as substrates (Table 1). The R_t of this product was higher than the substrate on HPLC and the R_f value of the product differed from any authentic anthocyanins used. Two intermediates, Cy 3G and cyanidin (Cy) were obtained by the controlled acid hydrolysis. The alkaline hydrolysis gave information for the presence of succinic acid in the product by TLC. All evidence obtained is compatible with the formation of cyanidin 3-succinylglucoside (Cy 3SG) by the acyltransferase. In addition, when pelargonidin 3-glucoside (Pg 3G) was used as the substrate, another product was found by HPLC and TLC analyses (Table 1). The product corresponds to pelargonidin 3-succinylglucoside (Pg 3SG) in a similar manner. On incubation of malonyl-CoA as an acyl donor and Cy 3G or Pg 3G, the co-chromatographic analysis on HPLC for the product indicated that each of these anthocyanins was acylated to form cyanidin- or pelargonidin 3-malonylglucoside.

The acyltransferase showed maximum activity at pH 7 with half maxima at pH 5.5 and 8.6. Calculated E_a was 69 and 176 kJ mol^{-1} following denaturation. These values are similar to those for malonyltransferase [2] and acetyltransferase [3]. The acylation was saturated with Cy 3G or Pg 3G at 500 μM, and with succinyl- or malonyl-CoA at 600 and 200 μM, respectively. The acylation reaction was not stimulated by any divalent ions tested, but rather inhibited by some of them; our results showed 71% inhibition by Cu^{2+} and about 30% by either Zn^{2+} or

Table 1. Chromatographic properties of anthocyanin produced by the acyltransferase reaction

Compound	R_f 100 in AHW	BAW	BuHCl	10F	EAA	ETN	R_t (min)
Reaction product **1***	23	52	30	22			29.1
Deacylated product of **1** and Cy 3G†	17	39	14	18			20.8
Hydrolysates of **1**†							
Hydrolysate I and Cy 3G	20						20.8
II and Cy	8						16.5
Reaction product **2***	60	62	77	56			31.7
Deacylated product of **2** and Pg 3G†	48	57	43	47			23.4
Hydrolysates of **2**†							
Hydrolysate I and Pg 3G†	35						23.4
II and Pg	14						20.0
Acylating acid of **1** or **2** and succinic acid†	69				87	16	
Related anthocyanins							
Cy 3G5G	30	21	5	36			15.9
Cy 3SG5G	57	41	20	60			21.9
Pg 3G5G	74	55	20	77			19.3
Pg 3SG5G	97	59	41	98			23.1

*Reaction products **1** and **2** refer to anthocyanins obtained using Cy 3G and Pg 3G as substrates, respectively.
†Deacylated products and controlled acid hydrolysates were analysed by co-chromatography.

Table 2. Specific activity of acyltransferase in cyanic and acyanic flowers of *Centaurea cyanus* cv Kanzaki-yae

Flower colour	Major anthocyanin (%)* Cy 3SG5G	Pg 3SG5G	Specific activity ($10^{-1} \times$ pkat μg^{-1}) Substrate: Succinyl-CoA Cy 3G	Succinyl-CoA Pg 3G	Malonyl-CoA Cy 3G	Malonyl-CoA Pg 3G
Blue	72	ND†	6.5	5.5	9.0	6.3
Pink	ND	69	6.2	4.6	8.3	6.5
White	ND	ND	0.1	< 0.1	0.1	0.1

*Content of anthocyanins extracted from flower petals with 50% HOAc was determined by HPLC.
†Not detected.

Hg^{2+}. No significant effect was found for enzyme inhibitors of diethylpyrocarbonate, diethyldithiocarbamate and *N*-ethylmaleimide.

Crude extracts were prepared from flower buds of blue, pink and white flowers in *Centaurea cyanus* cv Yaezaki and Kanzaki-yae. These preparations were examined for specific activity using various substrates, i.e. Cy 3G or Pg 3G as anthocyanins and succinyl-CoA or malonyl-CoA as acyl donors. The specific activity range was similar for the two cultivars. Thus, for simplicity, only the results for cv Kanzaki-yae are shown in Table 2. Regardless of the anthocyanin or acyl donor used as substrates, the specific activity was equally high in the extracts from cyanic flowers, irrespective of flower colour or type of major anthocyanin contained in the flowers. In contrast, the activity in the extracts from acyanic flowers was remarkably low (less than 2%) compared to cyanic flowers under any assay system. This result differs from that obtained in *Dendranthema* [2] and *Zinnia* [3], because a considerable amount of catalytically active acyltransferase was supposed to be synthesized even in acyanic flowers of these plants. It, therefore, implies that acylation of anthocyanins in flowers of *Centaurea* depends on the gene expression for the anthocyanidin biosynthesis as opposed to *Dendranthema* and *Zinnia*.

The Cy 3G and Pg 3G are nearly equivalent substrates for the acyltransferase (Table 2). In addition to these monoglucosyl anthocyanins, the 3,5-diglucosides of cyanidin and pelargonidin were examined for substrate specificity, and were not found to function as substrates. Cyanic flowers contain succinylated 3,5-diglucosides of anthocyanidin as a major anthocyanin [8–10] (Table 2).

Therefore, the results obtained above indicate that the acylation of anthocyanidin 3-glucosides preceeds 5-*O*-glucosylation in the flowers of this plant as reported in *Petunia hybrida* [11, 12] and *Zinnia elegans* [3].

Although a cyanidin 3-malonylglucoside was found in the leaves of blue flowered-*Centaurea cyanus* and cell cultures derived from stem of this plant [13], no malonylated anthocyanin has as yet been reported in its flowers. Thus, it is interesting that the acyltransferase extracted from cyanic flowers was found to catalyse the malonylation of anthocyanidin 3-glucosides at almost the same rate as succinylation. It, however, remains to be determined whether the catalytic activity of succinylation and malonylation comes from the same or different enzymes.

EXPERIMENTAL

Plant material. Flower buds of *Centaurea cyanus* cv Yaezaki and Kanzaki-yae were collected from a farm at Minami-Kyushu University.

Chemicals. Aliphatic CoA-esters were purchased from Sigma. Anthocyanins were isolated as described previously [14], and were further purified by preparative HPLC using an inertsil ODS-2 column (GL Science) and HOAc solvent system.

Enzyme preparation. Enzymes were prepared from the petals of flower buds essentially by the same methods described previously [2].

Enzyme assay. The standard reaction mixture contained, in a total volume of 100 μl, 10 μl enzyme extract, 500 μM Cy 3G or Pg 3G, 600 μM succinyl-CoA, and 80 μl of 0.1 M KPi buffer (pH 7) containing 10 mM L-cysteine. After incubation of the reaction mixture for 20 min at 35°, the reaction was stopped by the addition of 50 μl $CHCl_3$–MeOH (5% HCO_2H) (2:1), resulting in a Folch partition [15]. The upper phase was allowed to lyophilize before analysis.

Characterization of enzyme. Crude extracts from pink flowers of cv Kanzaki-yae were examined for optimum pH, E_a, and effects of divalent ions (1 mM) or enzyme inhibitors (1 mM) using succinyl-CoA and Pg 3G as substrates. The determination of optimum pH was performed using buffers of 0.1 M KPi (pH 5–7.5) or 0.1 M K_2Pi (pH 8–9.5, adjusted with 1 M KOH) containing 10 mM L-cysteine. Except for the saturation test for substrates, all measurements including the analysis of substrate specificity were made on reaction mixtures in saturated concentrations of each substrate.

Analytical methods. Anthocyanins were analysed by HPLC, and also in part by TLC. HPLC analysis was carried out by the same methods described in detail previously [3]. TLC was performed on micro-crystalline cellulose plates (Avicel SF, Funakoshi) in six solvents, AHW (HOAc–HCl–H_2O) (15:3:82), BAW (n-BuOH–HOAc–H_2O) (6:1:2), BuHCl (n-BuOH–2 M HCl) (1:1), 10F (HCO_2H–H_2O) (1:9), EAA (EtOAc–HOAc–H_2O) (3:1:1), ETN (EtOH–NH_4OH–H_2O) (16:1:3). Deacylation of the pigment purified from reaction products was conducted as described in ref. [14]. Controlled acid hydrolysis of the pigment was performed in 2 M HCl at 60° for 30 min. Hydrolysates were analysed by HPLC and TLC. The protein was determined by the method of ref. [16].

REFERENCES

1. Teusch, M. and Forkmann, G. (1987) *Phytochemistry* **26**, 2181.
2. Ino, I., Nishiyama, H. and Yamaguchi, M. (1993) *Phytochemistry* **32**, 1425.
3. Ino, I. and Yamaguchi, M. (1993) *Phytochemistry* **33**, 1415.
4. Kamsteeg, J., van Brederode, J., Hommels, C. H. and van Nigtevecht, G. (1980) *Biochem. Physiol. Pflanz.* **175**, 403.
5. Teusch, M., Forkmann, G. and Seyffert, W. (1987) *Phytochemistry* **26**, 991.
6. Strack, D. and Wray, V. (1994) in *The Flavonoids* (Harborne, J. B., ed.) pp. 1–23. Chapman & Hall, London.
7. Sulyok, G. and Laszlo-Bencsik, A. (1985) *Phytochemistry* **24**, 1121.
8. Takeda, K. and Tominaga, S. (1983) *Bot. Mag. Tokyo* **96**, 359.
9. Tamura, H., Kondo, T., Kato, Y. and Goto, T. (1983) *Tetrahedron Letters* **24**, 5749.
10. Takeda, K., Kumegawa, C., Harborne, J. B. and Self, R. (1988) *Phytochemistry* **27**, 1228.
11. Jonsson, L. M. V., Aarsman, M. E. G., van Diepen, J., de Vlaming, P., Smit, N. and Schram, A. W. (1984) *Planta* **160**, 341.
12. Heller, W. and Forkmann, G. (1988) in *The Flavonoids* (Harborne, J. B., ed.) pp. 399–425. Chapman & Hall, London.
13. Kakegawa, K., Kaneko, Y., Hattori, E., Koike, K. and Takeda, K. (1987) *Phytochemistry* **26**, 2261.
14. Yamaguchi, M., Terahara, N. and Shizukuishi, K. (1990) *Phytochemistry* **29**, 1269.
15. Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.