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CHROMATOGRAPHIC AND SPECTRAL CHARACTERIZATION OF 3′-GLYCOSYLATION IN ANTHOCYANIDINS

KUNIJIRO YOSHITAMA* and KENZO ABE†

* Department of Biology, Faculty of Science, Shinshu University, Matsumoto-city, Japan 390; † Chemical Institute, Faculty of Science, Tokyo Kyoiku University, Bunkyo-ku, Tokyo, Japan 112

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Abstract—The caffeyl ester of cyanidin 3,7,3′-triglucoside was isolated from red petals of garden cineraria, *Senecio cruentus*. Chromatographic and spectral characteristics of the anthocyanin as well as the products of deacylation and partial hydrolysis are described. The results with this pigment and a similar one based on delphinidin show that anthocyanins having a sugar residue at the 3′(or 5′)-position are characterized by the position of the visible max and by the high values for $E_{440}/E_{\text{vis max}}$ and $E_{\text{UV max}}/E_{\text{vis max}}$ when compared with other glycosides.

INTRODUCTION

Paper chromatography and absorption spectra are widely used for the characterization of anthocyanin pigments [1–4]. Most of the published data refer to the common anthocyanins which have sugars at the 3- or 3,5-positions. Only seven anthocyanins with sugars at the 7-position have been described [5–11] and one anthocyanin with a sugar at the 4′-position [12].

We have recently identified from blue flower of cineraria a caffeyl ester of delphinidin 3,7,3′-triglucoside, the first pigment to be found with a sugar at the 3′-position [13, 14]. We now report the identification of the related cyanidin derivative from the same plant [cf. 15] and collect together the relevant R_f and spectral data for anthocyanins with substitution at the 3′-position.

RESULT AND DISCUSSION

From red petals of cineraria, a purplish-red anthocyanin was isolated and purified by our standard procedure [13]. Upon acid hydrolysis, the pigment furnished cyanidin, glucose and caffeic acid, the molar ratio of cyanidin : glucose being 1:3. Although the amount of caffeic acid was not determined directly, there appear to be four molecules present since the $E_{\text{acid max}}/E_{\text{vis max}}$ ratio was 180%.

Deacylation gave a cyanidin triglucoside and this was characterized as the 3,7,3′-triglucoside by precisely the same procedures as used for the corresponding delphinidin derivative [13,14]. Substitution at the 7- and 3′-positions was confirmed by alkaline degradation of the methylated glucoside, which gave phloroglucinol monomethyl ether from the A-ring and *iso*-vanillic acid from the B-ring.

The effect of 3′-glycosylation on anthocyanidins already substituted in the 3-position is to produce a large hypsochromic shift in the visible spectrum. Thus, the 3,3′-diglucoside of cyanidin produced by partial hydrolysis of the 3,7,3′-triglucoside shows a hypsochromic shift of 5–9 nm, as compared with 3-, 3,5- and 3,7-diglucosides (Table 1). Moreover, there is a higher $E_{440}/E_{\text{vis max}}$ ratio in 3′-glucosides than in 3-, 5- or 7-glucosides. Similarly, the value of $E_{\text{UV max}}/E_{\text{vis max}}$ is higher.

While pelargonidin 3,7-diglycosides have different colours, spectra max and R_f values from related 3- and 3,5-glycosides [3], it is difficult to distinguish between 3-, 3,5- and 3,7-diglucosides of cyanidin by similar means [6, 9, 10]. However, the 3,3′-diglucoside of cyanidin is a distinctive orange-red compared to the red colour of 3-, 3,5- and 3,7-diglucoside and can be readily separated from them.

In the delphinidin series, there is also hypsochromic shift of the visible when the 3,7,3′-triglucoside is compared with the 3-rutinoside-5-glucoside due to the introduction of sugar into the 3′ (or 5′)-position.

As regards R_f values, glycosylation of the 3′-hydroxy tends to raise the R_f in butanolic solvents as compared with glycosylation elsewhere. This is true for mono or diglucosides. In general, it is possible to distinguish the 3′-glucoside from other glucosides by use of butanolic solvents. For example, while the 3,3′-diglucosides of cyanidin (and delphinidin) have similar R_fs to the corresponding 3,7-diglucosides in HOAc–HCl–H_2O (15:3:82) and 1% HCl, these anthocyanins can be clearly distinguished from the 3,7-diglucosides in butanolic solvents. During partial hydrolysis, it is the sugar at the 3′-position which is lost before those at the 3-, 5- or 7-positions.

No significant shift of absorption max occurs in the visible region when ordinary complex anthocyanins are deacylated. By contrast, with the complex anthocyanins from blue and red cineraria, a conspicuous hypsochromic shift occurs on deacylation, e.g. the visible max of complex cyanidin 3,7,3′-triglucoside being shifted from 533 nm to 513 nm following deacylation. It is noteworthy that the chloride of the complex anthocyanin

Table 1. Spectral and chromatographic data of various glucosides of cyanidin and delphinidin obtained by partial hydrolysis and deacylation of cineraria anthocyanins

Anthocyanins	Absorption spectra‡						R_fs (×100)§			
	λ_{max} (nm)			$E_{440}/E_{vis\,max}$ (×100)	$E_{UV\,max}/E_{vis\,max}$ (×100)	+$AlCl_3$	BAW	BuN	1%HCl	AAH
Cyanidin glucosides										
3-glucoside	530 (525)*	282		21 (22)*	30	+40	23 (38)*	23 (25)*	6 (7)*	24 (26)*
5-glucoside	534	278		9	17	+37	32	43	6	24
7-glucoside	531	278		19	43	+26	34	27	4	20
3'-glucoside	531	272		30	62	0	—	44	2	15
3-diglucoside (sophoroside)	528	282		25	51	+42	—	—	31	60
3,5-diglucoside	526	279		13	34	+34	14	4	13	40
3,7-diglucoside	524 (525)†	284		21 (22)†	49	+36 (11)†	11 (20)†	3 (5)†	20 (17)†	49 (52)†
3.3'-diglucoside	519	279		35	61	0	16	7	20	49
3-sophoroside-5-glucoside	525	279		12	23	+43	15	6	53	75
3,7,3'-triglucoside	513	281		34	67	0	8	1	47	67
3,7,3'-triglucoside (acylated)	533	324	286	25	—	0	16	16	3	15
3,7,3'-triglucoside‖ (acylated, in 50% ethanol)	588	544 309	510	—	—	0	—	—	--	--
Delphinidin glucosides										
3,5-diglucoside	538	276		10	40	+50	8	3	7	28
3,7-diglucoside	537	283		15	—	+46	6	3	11	39
3,3'-diglucoside	531	279		25	51	+48	10	7	11	39
3-rutinoside-5-glucoside	540	281		10	56	+41	12	5	30	54
3,7,3'-triglucoside	525	282		22	—	0	4	1	34	58
3,7,3'-triglucoside (acylated)	550	328	290	21	—	0	9	22	2	11
3,7,3'-triglucoside‖ (acylated, in 50% EtOH)	630	580 315	540	—	—	0	—	—	—	—

* Data according to Harborne [1, 2]. † Data of Birkofer [6]. ‡ Measured in 0.01% MeOH–HCl. § Determined by PC on Toyo No. 51 at 25 ± 1°. ‖ Measured in 50% EtOH. Solvent abbreviations: BAW: *n*-BuOH–HOAc–H_2O (4:1:5); BuN: *n*-BuOH–2N HCl (1:1); 1% HCl: HCl–H_2O (3:97); AAH: HOAc–HCl–H_2O (15:3 82).

from red cineraria exhibits three absorption peaks (Table 1) and retains its stable colour in neutral solution, as has been previously described for the blue pigment isolated from Chinese bellflower [16] and also from blue cineraria [13]. Therefore, it seems that in these pigments the unusual stability of the anthocyanin colour may possibly reside in some interaction between the anthocyanin moiety and the complexing organic acid(s) present.

EXPERIMENTAL

Isolation of the anthocyanins. Red (1.5 kg) and purplish–blue (1.5 kg) petals of cineraria were dried over $CaCl_2$ *in vacuo*, and extracted with Me_2CO–EtOH–H_2O (4:1:4). The extract was purified by the same procedure as described previously [13]. Finally, the pigments were obtained as blue and purple granules weighing 300 and 540 mg, respectively.

Quantitative analysis of cyanidin 3,7,3'-triglucoside. This was obtained by deacylation of the original complex anthocyanin and purified as already described [14]. For quantitative analysis, it was dissolved in 6 N HCl, boiled for 3 min and cooled quickly in H_2O. The components, cyanidin and glucose, were partitioned as usual into *iso*-amyl alcohol containing 1% of conc HCl and H_2O, respectively. Cyanidin was determined spectrophotometrically using λ_{max} 550 nm and sugar was determined by the TTC method using characteristic absorption at 480 nm for glucose [17]. (Found: cyanidin, 36.9%; glucose, 60.8%. Cyanidin-triglucoside requires: cyanidin, 39.9%; glucose, 66.8%.)

Cyanidin 3'-monoglucoside. This glucoside was obtained as an intermediary product of partial hydrolysis, and was separated by PC. Further purification was made in *n*-BuOH–2N HCl (1:1) and HOAc–HCl–H_2O (15:3:82). The λ_{max} of this pigment did not shift on the addition of $AlCl_3$, indicating substitution in the 3'- or 4'-position. Partial hydrolysis gave cyanidin alone without the production of intermediate pigment; accordingly, the pigment must be monoglucoside. Since the original triglucoside was established with 3,7,3'-trisubstitution, this pigment must be the 3'-monoglucoside of cyanidin. In the same way, cyanidin 3- and 7-monoglucosides were obtained and identified. The cyanidin 3-glucoside was further identified by comparison with authentic pigment from *Cinnamonum japonica* [18].

Cyanidin 3,3'-diglucoside. This glucoside was also obtained by partial hydrolysis. Addition of $AlCl_3$ to a solution of the glucoside gave no shift, so that the 3'-OH is glucosylated. This anthocyanin is a dimonside since partial hydrolysis yielded cyanidin 3-monoglucoside, cyanidin 3'-monoglucoside and cyanidin; this is also supported by R_f values (Table 1). Consequently, the anthocyanin is confirmed as cyanidin 3,3'-diglucoside. The alternative 3,4'-diglucoside structure is precluded by comparison with the data of Hedin *et al.* [12], who record a visible max for the 3,4'-diglucoside at the same position as the 3,5-diglucoside.

Cyanidin 3,7-diglucoside. Addition of $AlCl_3$ brought about a bathochromic shift in visible max, indicating the presence of a free *ortho*-dihydroxyl grouping. That the value of $E_{440}/E_{vis\,max}$ is 21% shows that there is a free OH in the 5-position. Partial hydrolysis showed this pigment to be a diglucoside, since the 3- and 7-glucosides of cyanidin were detected. The anthocyanin is thus the 3,7-diglucoside of cyanidin.

3,7- and 3,3'-diglucoside of delphinidin. The glucoside structure of these anthocyanins was determined in the same was as for the corresponding glucosides of cyanidin. Further discrimina-

tion of both anthocyanins was made by their spectral characteristics, e.g., visible max of 3,3′-diglucoside lying in a shorter wavelength region than that of 3,7-diglucoside.

PC, TLC and absorption spectra. All R_f-values were measured on Toyo No. 51 filter paper, a grade corresponding to Whatman No. 1 paper or Shleicher and Shüll 2043b MGL. Avicel TLC plates were also used. Absorption spectra were taken with Shimazu UV-200 double beam spectrophotometer.

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