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BLUEING OF SEPAL COLOUR OF *HYDRANGEA MACROPHYLLA*

KÔSAKU TAKEDA, MIYUKI KARIUDA and HIDEAKI ITOI

Department of Biology, Tokyo Gakugei University, Koganei, Tokyo 184, Japan

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Key Word Index—*Hydrangea macrophylla*; Saxifragaceae; blueing; anthocyanin; 3-caffeoylquinic acid; aluminium.

Abstract—Blue and red sepals of *Hydrangea macrophylla* were quantitatively analyzed for aluminium, anthocyanin (delphinidin 3-glucoside) and copigments (caffeoyl- and *p*-coumaroylquinic acids). All the blue sepals examined contained both Al and copigments (especially 3-caffeoylquinic acid) in considerable amounts. In *in vitro* experiments using 3- and 5-caffeoylquinic acids, Al and delphinidin 3-glucoside, it was shown that 3-caffeoylquinic acid and Al formed a blue complex with the anthocyanin. Absorption spectra of the blue complex were practically identical with those of the blue solutions obtained from blue hydrangea sepals by extraction with 4 M NaCl. In contrast, 5-caffeoylquinic acid (chlorogenic acid) which was also present in hydrangea sepals gave only a red–purple colour with Al and the anthocyanin. Neither 3-caffeoylquinic acid nor Al independently produced blue colour when mixed with the anthocyanin in the mole ratios of 1–30, this being the range that the compounds were found in blue sepals. These results suggest that blue colour of hydrangea sepals is due mainly to the blue complex of delphinidin 3-glucoside–aluminium–3-caffeoylquinic acid. The role of aluminium may be to stabilize an interaction between the quinic ester and the anthocyanin.

INTRODUCTION

The participation of aluminium in the blueing of hydrangea sepals is well known [1–5]. We have reported recently some qualitative observations indicating that blueing of the hydrangea anthocyanin, delphinidin 3-glucoside, can be caused also by copigments found in the blue sepals and identified as 3-caffeoyl- and 3-*p*-coumaroylquinic acids [6]. 5-Caffeoylquinic acid (chlorogenic acid) also present was less effective as a copigment since it produced a purple colour rather than blue [6]. To clarify the problem of blueing in sepals of *Hydrangea macrophylla*, the quantitative analyses of Al, the copigments and the anthocyanin in blue and red sepals were carried out and on the basis of the analytical results the relationship between those factors and blue colouration was investigated.

RESULTS AND DISCUSSION

Contents of anthocyanin, caffeoyl- and p-coumaroylquinic acids, and Al in blue and red hydrangea sepals

The blue sepals examined contained much more Al than red sepals, so confirming the previous findings of Allen [1, 2] and Chenery [3] (Table 1). Blue sepals also contained more 3-caffeoyl- and 3-*p*-coumaroylquinic acids but less 5-caffeoylquinic acid than red sepals; the amounts of delphinidin 3-glucoside were similar in both colour types. In particular 3-caffeoylquinic acid, known to have a blueing effect on the hydrangea anthocyanin [6] occurred in substantial amounts in blue sepals, whereas it was scarcely detectable in red sepals (except R5 and R6). Of the compounds described above, anthocyanin pigment is localized generally in a layer of cells just beneath the epidermis of the upper and lower surfaces of the sepals but it is uncertain whether Al and copigments are also localized in the pigmented cells. Accordingly atomic or molecular ratios of Al or copigments to anthocyanin in the cell sap of hydrangea sepals may be lower than the values shown in Table 1.

In view of these results, the effects of Al and copigments on anthocyanin colour were examined independently and in mixtures, at concentrations up to 30 times molar excess over anthocyanin.

The separate effects of 3- and 5-caffeoylquinic acids, and Al on the absorption spectra of delphinidin 3-glucoside

The effects of 1–30 molecular equivalents of 3- or 5-caffeoylquinic acids or $AlCl_3$ on the absorption spectra of delphinidin 3-glucoside were evaluated. Since pH values of blue and red solutions [6] which were obtained from blue and red sepals by extraction with 4 M NaCl (pH 4.50) were 3.50–4.10, the cell sap of hydrangea sepal seemed to be fairly acidic, and thus the *in vitro* experiments were carried out at pH 3.70. As shown in Table 2, additions of $AlCl_3$ to solutions of anthocyanin resulted in marked increases in absorbance and bathochromic shifts. However, the colours of the solutions were reddish purple. 3-Caffeoylquinic acid did not give a blue solution when present at molar ratios between 1–30 suggesting that a higher molar ratio is necessary to produce the blueing previously observed with this compound [6]. Indeed, under the conditions employed, the copigmentation effects of 3- and 5-caffeoylquinic acids were very small and almost the same as each other. The results showed that none of these compounds independently changed the colour of the solution of delphinidin 3-glucoside to blue when present within the range of molecular ratios (1–30) found to occur in blue hydrangea sepals.

Table 1. Contents of delphinidin 3-glucoside, caffeoyl and *p*-coumaroyl quinic acids, and aluminium in blue and red hydrangea sepals

	Content in fresh sepals (ppm)†					Molar or atomic ratio†				
Material*	Del-3G	Al	3-Caf	5-Caf	3-*p*C	Del-3G	Al	3-Caf	5-Caf	3-*p*C
B1	285	150	1725	2479	396	1	9.8	8.6	12.3	2.1
B2	325	118	2731	2983	726	1	6.7	11.9	13.0	3.3
B3	312	73	1983	2440	312	1	4.4	9.0	11.1	1.5
B4	353	126	2154	2683	320	1	6.6	8.6	10.8	1.3
B5	277	111	1840	3784	527	1	7.5	10.0	19.3	2.8
B6	454	91	1581	2542	500	1	3.7	4.9	7.9	1.6
B7	416	122	1690	2213	340	1	5.4	5.7	7.5	1.2
Mean	346	113	1958	2732	446	1	6.3	8.4	11.7	2.0
R1	295	32	—	7150	404	1	2.0	—	34.3	2.0
R2	390	29	339	8493	201	1	1.4	1.2	30.8	0.8
R3	372	39	—	7561	362	1	1.9	—	28.8	1.4
R4	405	43	—	8049	530	1	2.0	—	28.1	1.9
R5	339	26	3592	4592	108	1	1.4	15.0	19.2	0.5
R6	487	17	2374	3978	416	1	0.7	6.9	11.6	1.3
R7	406	40	—	8548	599	1	1.8	—	29.8	2.2
Mean	385	32	901	6910	374	1	1.6	3.3	26.1	1.4

*B1–B7: Blue sepals; R1–R7: red sepals.
†Del-3G: Delphinidin 3-glucoside; Caf: caffeoyl quinic acid; *p*C: *p*-coumaroyl quinic acid; —, not detected.

Table 2. The separate effects of 3- and 5-caffeoylquinic acids and aluminium on the λ_{max} and absorbance of delphinidin 3-glucoside in 0.05 M acetate buffer (pH 3.70)

	Delphinidin 3-glucoside (1×10^{-4} M) mixed with					
Molar ratio of added compound to delphinidin 3-glucoside	$AlCl_3$		3-Caf†		5-Caf†	
	λ_{max} (nm)	A* at λ_{max}	λ_{max} (nm)	A* at λ_{max}	λ_{max} (nm)	A* at λ_{max}
0:1	528	0.060	528	0.060	528	0.060
1:1	568	0.198	528	0.157	528	0.158
5:1	572	0.412	528	0.162	—	—
10:1	572	0.510	530	0.172	528	0.170
20:1	572	0.590	—	—	530	0.203
30:1	572	0.605	532	0.230	532	0.232

*Light path length of 3 mm.
†Caf, Caffeoyl quinic acid; —, not determined.

Complexes of delphinidin 3-glucoside with Al *and copigments*

The effects of 3- and 5-caffeoylquinic acids on the spectrum of delphinidin 3-glucoside (1×10^{-4} M) at pH 3.70 in the presence of $AlCl_3$ (1×10^{-4} M) are summarized in Table 3. 3-Caffeoylquinic acid showed a remarkable blueing effect. The bathochromic shifts and absorbances increased with increasing concentrations of 3-caffeoylquinic acid. In contrast, 5-caffeoyl quinic acid and Al showed only small effects, giving reddish-purple colours.

The effect of Al on the spectrum of delphinidin 3-glucoside (1×10^{-4} M) containing 10 molar equivalents of 3- or 5-caffeoylquinic acid was then determined. In the presence of 3-caffeoylquinic acid, increasing Al ratios produced progressively increasing effects and the colours of the solutions became blue on adding Al more than one atomic equivalent as shown in Table 4. The blue colours of the solutions were stable within the range of pH 3.23–5.22. The solutions became reddish purple at pH 2.98 and red at pH values lower than 2.65. With 5-caffeoylquinic acid, the increases in intensity and bathochromic shifts of the visible maxima were smaller than with 3-

Table 3. The effects of 3- and 5-caffeoylquinic acids on the λ_{max} and absorbance of mixtures of delphinidin 3-glucoside (1×10^{-4} M) and $AlCl_3$ (1×10^{-4} M) in 0.05 M acetate buffer (pH 3.70)

Molar ratio of copigment to delphinidin 3-glucoside	Delphinidin 3-glucoside (1×10^{-4} M) + $AlCl_3$ (1×10^{-4} M) mixed with					
	3-Caf†			5-Caf†		
	λ_{max} (nm)	A* at λ_{max}	Colour of the solution	λ_{max} (nm)	A* at λ_{max}	Colour of the solution
0:1	568	0.198	Reddish purple	568	0.198	Reddish purple
0.5:1	576	0.422	Purplish blue	568	0.237	Reddish purple
1:1	577	0.510	Blue	568	0.242	Reddish purple
2:1	581	0.583	Blue	568	0.204	Reddish purple
5:1	584	0.680	Blue	564	0.190	Reddish purple
10:1	585	0.725	Blue	560	0.180	Reddish purple
20:1	587	0.750	Blue	554	0.175	Pink
30:1	588	0.790	Blue	544	0.190	Pink

*Light path length of 3 mm.
†Caf, Caffeoyl quinic acid.

Table 4. Effects of aluminium on the λ_{max} and absorbance of delphinidin 3-glucoside in the presence of excess of 3- or 5-caffeoylquinic acid in 0.05 M acetate buffer (pH 3.70)

Molar ratio of $AlCl_3$ to delphinidin 3-glucoside	Delphinidin 3-glucoside (1×10^{-4} M) in the presence of					
	3-Caf† (10 Mole Equiv.) + $AlCl_3$			5-Caf† (10 Mole Equiv.) + $AlCl_3$		
	λ_{max} (nm)	A* at λ_{max}	Colour of the solution	λ_{max} (nm)	A* at λ_{max}	Colour of the solution
0:1	530	0.172	Pink	528	0.170	Pink
0.5:1	586	0.493	Purplish blue	—	—	
1:1	586	0.692	Blue	559	0.198	Reddish purple
2:1	587	0.780	Blue	569	0.290	Reddish purple
5:1	585	0.942	Blue	572	0.440	Reddish purple
10:1	584	0.982	Blue	572	0.598	Reddish purple
20:1	582	0.976	Blue	572	0.600	Reddish purple
30:1	581	0.990	Blue	572	0.620	Reddish purple

*Light path length of 3 mm.
†Caf, Caffeoyl quinic acid.

caffeoylquinic acid and the colours of the solutions were reddish purple rather than blue. Even in the presence of a larger amount, i.e. 30 molecular equivalents of the 5-ester, the results were similar to those above and the solutions were reddish purple on adding 1–30 atomic ratios of Al.

Finally, the effect of Al was investigated on the colour of delphinidin 3-glucoside containing a mixture of 3- and 5-caffeoylquinic acids in molar ratios approximating to the mean values found in blue sepals (Table 1). As shown in Table 5 the colours of these model systems became blue in the presence of one or more atomic equivalents of Al. It is noteworthy that the absorption spectra of the blue solutions were practically identical with those of the blue solutions (maxima, within the range 582–585; pH 3.75–4.10), which were obtained from blue hydrangea sepals by extraction with 4 M NaCl [6].

The results show that although Al has a greater effect than 3-caffeoylquinic acid at the molar ratios used, the presence of both compounds is necessary to achieve a full blue colour. Thus, it would appear reasonable to speculate that in blue hydrangea sepals 3-caffeoyl (and probably 3-*p*-coumaroyl)quinic acid forms a blue complex with Al and delphinidin 3-glucoside. Since addition of 3-caffeoyl- or 3-*p*-coumaroylquinic acid alone in high concentration to the hydrangea anthocyanin causes a colour change from red to blue [6], the blueing can be ascribed to a molecular interaction between copigments and anthocyanin, and thus the role of Al would be to stabilize the interaction. In complexes Al may conjugate with the carboxyl residue of the quinic ester and with an *ortho*-dihydroxyl of the B-ring of delphinidin 3-glucoside (quinoidal base). On the other hand, in red sepals the

Table 5. Effects of aluminium on the λ_{max} and absorbance of delphinidin 3-glucoside in the presence of 3- and 5-caffeoylquinic acids in 0.05 M acetate buffer (pH 3.70)

Molar ratio of $AlCl_3$ to delphinidin 3-glucoside	Delphinidin 3-glucoside (1×10^{-4} M) + 3-Caf† (8 mole equiv.) + 5-Caf† (12 mole equiv.) + $AlCl_3$		
	λ_{max} (nm)	A* at λ_{max}	Colour of the solution
0:1	532	0.128	Pink
0.1:1	542	0.150	Purplish red
0.2:1	574	0.210	Reddish purple
0.5:1	583	0.372	Purplish blue
1:1	585	0.505	Blue
2:1	585	0.662	Blue
5:1	585	0.720	Blue
10:1	583	0.739	Blue
20:1	582	0.746	Blue
30:1	582	0.748	Blue

*Light path length of 3 mm.
†Caf, Caffeoyl quinic acid.

atomic or molecular ratios of Al and/or the copigments (3-esters) in the pigmented cells may be too low to produce a blue colour. The formation of copigment–aluminium–anthocyanin complexes of similar type has been described previously but involved 5-caffeoylquinic acid (chlorogenic acid); 3-caffeoylquinic acid was not examined [7].

EXPERIMENTAL

Materials. Blue and red sepals of *Hydrangea macrophylla* were collected at various places in Tama district of Tokyo.

Contents of anthocyanin, copigments and aluminium. Anthocyanin: Pigment was thoroughly extracted with 1% MeOH–HCl from fresh sepals and determined photometrically as its chloride by measuring absorbance at 540 nm. Al: Fresh sepals were homogenized with 1% HCl in a mortar. After repeating the extraction several times, the extract was filtered and the filtrate was adjusted to a definite volume. The quantity of Al was determined by inductively coupled plasma (ICP) emission spectrometry. Copigments: Fresh sepals were extracted with 70% EtOH under reflux for 15 min. After repeating this extraction 4 times, the combined extract was filtered and the filtrate was evaporated to dryness. The residue was dissolved in H_2O (5 ml) and aliquot (0.1 ml) was applied to mass PC, in which descending method was used with *n*-BuOH–HOAc–H_2O, 4:1:2 (BAW). Three bands showing blue (3-*p*-coumaroylquinic acid) and yellow (3- and 5-caffeoylquinic acids) fluorescences under UV in NH_3 were separated and cut off. Each compound was eluted from the band with 0.05 M acetate buffer (pH 4.80) and determined spectrophotometrically by measuring absorbance at 310 nm (*p*-coumaroylquinic acid) or 320 nm (caffeoylquinic acid). On PC and cellulose TLC with 2% HOAc and BAW, each compound isolated by the above procedure showed a single spot which was identical with that of authentic ester. These analyses were repeated at least twice on inflorescences from a single plant and the mean contents are shown in Table 1.

Effects of copigments and Al *on the absorption spectra of delphinidin 3-glucoside.* 3-Caffeoylquinic acid was obtained by interconversion of 5-caffeoylquinic acid (Sigma) as described previously [6]. $AlCl_3$ and/or copigments solns whose pH values were previously adjusted to 3.7 with solid Na_2CO_3 or aq. NaOH soln were added in various molar (atomic) ratios to the solns of authentic delphinidin 3-glucoside. After standing for 1 hr at room temp., absorption spectra were measured on a spectrophotometer (light path length of 3 mm) and then pH values of the solns were measured. Final concn of anthocyanin in the solns was 1×10^{-4} M in 0.05 M acetate buffer of pH 3.70.

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