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# COPIGMENTS IN THE BLUEING OF SEPAL COLOUR OF *HYDRANGEA MACROPHYLLA*

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**Key Word Index**—*Hydrangea macrophylla*; Saxifragaceae; copigmentation; blueing; sepal colour; anthocyanin; 3-*p*-coumaroylquinic acid; 3-caffeoylquinic acid.

**Abstract**—From blue sepals of *Hydrangea macrophylla*, copigments which show a blueing effect on the hydrangea anthocyanin were isolated and identified as 3-*p*-coumaroylquinic acid and 3-caffeoylquinic acid. 5-Caffeoylquinic acid (chlorogenic acid) which was also found in the blue sepals, however, did not show such a blueing effect though it acted as a copigment. Likewise, the 4-esters of *p*-coumaroyl- and caffeoylquinic acids (not found in sepals) produced purple rather than blue colours. The facts suggest that the stereostructures of 3-*p*-coumaroyl- and 3-caffeoylquinic acids are effective for molecular interaction between the *p*-coumaroyl or caffeoyl residue in the compounds and the anthocyanin. The anthocyanin in red and blue sepals of hydrangea was confirmed to be delphinidin 3-monoglucoside.

## INTRODUCTION

Allen [1, 2] and Chenery [3] reported that blueing in hydrangea sepals was achieved by the complexing of anthocyanin with aluminium. Later, Asen *et al.* compared phenolic compounds in red and blue hydrangea sepals [4] and determined the absorption spectra of flavonoid pigments from sepals with or without aluminium [5]. The anthocyanin in sepals of hydrangea has been examined by several investigators. Lawrence *et al.* [6] identified the anthocyanin as delphinidin 3-glucoside from the results of colour reactions and distribution ratios. Hayashi *et al.* [7] reported that the pigment was a delphinidin 3-glycoside. On the other hand, Asen *et al.* [4] found a delphinidin arabinoglucoside in red and blue sepals. Thus, it seemed necessary to reexamine the nature of the anthocyanin in red and blue sepals.

The purpose of this investigation was to clarify whether aluminium is the sole factor involved in the blueing of hydrangea sepals, and to determine the chemical nature of the anthocyanin.

## RESULTS AND DISCUSSION

### *Anthocyanin in blue and red sepals*

On complete acid hydrolysis, anthocyanins isolated from blue and red sepals gave delphinidin and glucose. Under mild acid hydrolysis [8], the pigments were hydrolysed directly into delphinidin and no intermediary glycosides could be detected. Moreover, both anthocyanins gave rise to glucose by oxidative degradation with hydrogen peroxide [9]. $R_f$-values and absorption spectra of the pigments were identical with those of authentic delphinidin 3-monoglucoside.

### *Presence of copigments in blue sepals*

For the extraction of genuine pigment from hydrangea sepals, aqueous 4 M NaCl solution was used [10]. When sepals were homogenized with or without water in a mortar, however, the pigment was decomposed gradually and became brown, due to oxidation. By extraction with 4 M NaCl, blue ($\lambda_{max}$ 585 nm, pH 3.75) and red ($\lambda_{max}$ 538 nm, pH 3.78) solutions were obtained from blue and red sepals of hydrangea respectively. The blue solution was applied to a Sephadex column and fractionated (Nos. 1–30). During downward flow the blue colour soon changed to red. Each fraction was evaporated to dryness and the residue dissolved in water (1 ml). All fractions were colourless except red fractions of Nos. 18 and 19 which contained anthocyanin. After adjusting pH of each fraction to 4.0, a small amount of red aqueous solution of delphinidin 3-glucoside was added to each fraction, whereupon fraction Nos. 4–6 formed stable blue colours and fraction Nos. 3, 7 and 8 became purplish-blue. However, in other fractions the colour of the added anthocyanin soon faded. The red colour of fraction Nos. 18 and 19 was also unstable. Since the blueing effect of fractions Nos. 4–6 was not lost even after passing through a cation exchanger column, it is unlikely that metals such as Al and Fe would participate in this phenomenon. In a parallel experiment using red sepals, such a blueing phenomenon was not observed. These facts suggest that a copigment was present in blue sepals of hydrangea.

### *Properties of the copigments*

As described in the Experimental, three compounds, $A_1$, $A_2$ and B, which show a blueing effect on anthocyanin pigment were isolated. On alkaline hydrolysis, $A_1$ and $A_2$ gave rise to *p*-coumaric acid, while B gave caffeic acid. On enzymic hydrolysis with tannase, $A_1$ and $A_2$ yielded *p*-coumaric acid and quinic acid, and B gave caffeic acid and quinic acid. From the chromatographic comparison with the three (3-, 4- and 5-) isomeric esters, it became evident that $A_1$ and $A_2$ are *trans*- and *cis*-isomers of 3-*p*-coumaroylquinic acid respectively and that B is 3-

Table 1. Copigmentation test with 3-, 4- and 5-esters of *p*-coumaroyl- and caffeoylquinic acids

| | Delphinidin 3-glucoside (pH 4.0) mixed with† | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | 3-pC | 4-pC | 5-pC | 3-Caf | 4-Caf | 5-Caf |
| Colour of the solution | Red‡ | Blue | Purple | Purple | Blue | Purple | Purple |
| $\lambda_{max}$ (nm) | 535–538 | 585 | 564 | * | 585 | * | 548 |

*Not determined.
†pC: *p*-Coumaroylquinic acid; Caf: Caffeoylquinic acid.
‡Unstable.

caffeoylquinic acid. When an aqueous solution (pH 4.0) of each copigment was mixed with a red aqueous solution of delphinidin 3-glucoside, blue colours were produced and a bathochromic shift as well as a large increase in absorbance was observed: the $\lambda_{max}$ changed from 535–538 to 585 nm. The blue colour of the solutions was very stable and absorption spectra were practically identical with that of the blue solution (4 M NaCl) from hydrangea sepals. Chlorogenic acid (5-caffeoylquinic acid) was also isolated by us from blue sepals, but did not show any blueing effect. The presence of 3-caffeoylquinic acid and chlorogenic acid in hydrangea sepals has been already reported by Asen *et al.* [4].

*Copigmentation with* p-*coumaroyl- and caffeoylquinic acids*

To confirm the above results, copigmentation tests were carried out using 3-, 4- and 5-isomers of *p*-coumaroyl- and caffeoylquinic acids which were prepared from authentic esters. As shown in Table 1, all compounds showed a copigmentation effect on the anthocyanin, i.e. they changed the red colour of the aqueous anthocyanin solution to purple or blue. But only the 3-esters gave blue colours. The 4-ester gave larger bathochromic shift than the 5-ester. These results suggest that only the 3-esters are effective for blueing. The blueing effect of these compounds can be ascribed to the interaction between anthocyanin and the *p*-coumaroyl or caffeoyl residue of the esters as in the case of gentiodelphin [11, 12] and platyconin [13, 14].

## EXPERIMENTAL

*Anthocyanin.* Anthocyanins were isolated from blue and red sepals (dried 50 g of each) of *Hydrangea macrophylla* by extraction with 1% MeOH–HCl, precipitation as lead salt, conversion into chloride, precipitation with $Et_2O$, and purification by Sephadex LH 20 column using 50% EtOH as eluant. Each anthocyanin was purified by cellulose column chromatography with *n*-BuOH–HOAc–$H_2O$, 4:1:2 (BAW). Final purification was attained by cellulose TLC with HOAc–HCl–$H_2O$, 3:1:8 (AHW). $R_f$s of both pigments and delphinidin 3-monoglucoside were 0.18 (red sepals), 0.18 (blue sepals), 0.18 in *n*-BuOH–HCl–$H_2O$, 7:2:5; 0.25, 0.26, 0.26 in AHW.

*Isolation of copigments.* Fresh blue sepals (60 g) were homogenized in a mortar with aq. soln of 4 M NaCl (30 ml). The homogenate was strained through a gauze and the filtrate was centrifuged at 10000 *g* for 40 min. The blue supernatant (6 ml) was applied on Sephadex LH 20 column (2.7 × 20 cm) using $H_2O$ as eluant and fractionated (30 ml each, Nos. 1–30). Fractions Nos. 4–6 which contained copigment were concentrated. The soln was then passed through an ion exchange column (Dowex 50W, H-form, 0.8 × 16 cm) and the effluent was evaporated to dryness. The residue was then applied to a Sephadex LH 20 column (2.7 × 40 cm), again using 0.2% HOAc. Further purification of the copigment was carried out by mass PC using 2% HOAc as solvent. Three bands showing blue ($A_1$ and $A_2$) and yellow (B) fluorescences under UV in $NH_3$ were separated on the chromatograms. Finally, each compound was purified by mass PC with BAW. During the process, chlorogenic acid was also isolated.

*Alkaline hydrolysis of the copigments.* Copigments were treated with 2 N aq. NaOH for 45 min at room temp. in $N_2$ and acidified with 5% HCl. The organic acids were extracted with $Et_2O$ and examined by PC using BAW, 1% HCl and $C_6H_6$–HOAc–$H_2O$, 6:7:3 as solvents.

*Enzymic hydrolysis with tannase.* To an aq. soln (0.5 ml) of each copigment, tannase was added with a few drops of toluene and the soln was kept at 30° for 24 hr. The reaction mixture was shaken with $Et_2O$. $Et_2O$ and aq. layers were evaporated and applied to PC. For PC of organic acids in $Et_2O$, the same solvents as above were used. From both aq. layers only one spot of quinic acid was detected by using nitroprusside reagent [15]. $R_f$s of the products in aq. layers from $A_1$, $A_2$ and B, quinic acid and shikimic acid were: 0.28 ($A_1$), 0.28 ($A_2$), 0.28 (B), 0.28 and 0.49 in BAW; 0.42, 0.42, 0.42, 0.42 and 0.48 in PhOH–$H_2O$, 3:1; 0.38, 0.38, 0.38, 0.38 and 0.49 in EtOAc–$HCO_2H$–$H_2O$, 10:2:3.

*PC and UV of the copigments.* $R_f$s of $A_1$, $A_2$, 3-, 4- and 5-*p*-coumaroylquinic acids, B, 3-, 4- and 5-caffeoylquinic acids were: 0.72, 0.73, 0.72, 0.78, 0.83, 0.51, 0.50, 0.58, 0.64 in BAW; 0.68 (*trans*-isomer) and 0.80 (*cis*-isomer), 0.68 and 0.80, 0.68 and 0.80, 0.61 and 0.77, 0.63 and 0.77, 0.59, 0.59, 0.58, 0.52 in 2% HOAc. $\lambda_{max}^{MeOH}$ nm: $A_1$ 312, $A_2$ 312, 4-*p*-coumaroylquinic acid 312, B 330, 5-caffeoylquinic acid 330.

3-, 4- *and 5-Esters of* p-*coumaroyl and caffeoyl quinic acids.* 3- and 5-*p*-Coumaroylquinic acids were obtained by interconversion of authentic 4-*p*-coumaroylquinic acid according to the method of Haslam *et al.* [16]. 3- and 4-Caffeoylquinic acids were prepared from chlorogenic acid (Sigma) after the method of Hanson [17]. Isolation of 3-, 4- and 5-isomers was carried out by silica gel column chromatography [18].

*Copigmentation test.* To an aq. soln of each ester (pH 4.0) red aq. soln of authentic delphinidin 3-glucoside was added.

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