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ACYLATED ANTHOCYANINS IN A HYBRID *VITIS* VARIETY

RONALD A. FONG*, A. DINSMOOR WEBB and RICHARD E. KEPNER†

Departments of Chemistry and of Viticulture and Enology, University of California, Davis, CA 95616, U.S.A.

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Abstract—The anthocyanin skin pigments of the hybrid grape variety Royalty have been reexamined using isolation and chromatographic techniques designed to avoid degradation of the more labile pigments. Acetic acid has been identified as an acylating acid in acylated anthocyanin-3,5-diglucosides. The position of acylation of the malvidin-3,5-digucoside *p*-coumarate has been shown to be on the C-6 OH of the 3-0 glucose moiety.

INTRODUCTION

ANTHOCYANIN 3,5-diglucosides, although widespread generally,[1] do not occur in *Vitis vinifera* varieties. They are found, however, in *riparia* and *rupestris* varieties[2,3] and the many hybrids from crosses with *vinifera* varieties. The acylated anthocyanin diglucosides have not been as thoroughly investigated as the monoglucosidic pigments, probably due in part to the extremely complex pigment mixtures present in the grape hybrids. Chen and Luh[4] reported six diglucoside and seven monoglucoside anthocyanin pigments, some of which are acylated with *p*-coumaric or caffeic acids, in the skins of Royalty grapes, a hybrid of Alicante Ganzin and Trousseau; no attempt was made to determine the exact location of these acids.

We have shown that malvidin-3-monoglucoside *p*-coumarate from *V. vinifera* variety Tinta Pinheira has the acid at the C-6 OH of the glucose.[5] It has also been demonstrated that acetic acid is a major acylating acid in grape anthocyanin monoglucosides.[6,7] This paper reports that acetic acid also occurs acylating anthocyanin 3,5-diglucosides in the variety Royalty. The position of acylation in malvidin-3,5-diglucoside coumarate from Royalty grapes has also been determined.

RESULTS AND DISCUSSION

Earlier investigations in our laboratory[6] demonstrated that acetylated anthocyanins are extremely labile and decompose rapidly in the presence of mineral acids. The

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* Present address: Rohm and Hass Chemical Co., 5000 Richmond Street, Philadelphia, PA 19137, U.S.A.

† To whom inquiries should be addressed (Department of Chemistry).

[1] HARBORNE, J. B. *Comparative Biochemistry of the Flavonoids*, Academic Press, New York (1967).

[2] RIBÉREAU-GAYON, J., SUDRAUD, P. and DURQUETY, P. M. (1955) *Rev. Gén. Botany* **62**, 667.

[3] RIBÉREAU-GAYON, J. and RIBÉREAU-GAYON, P. (1958) *Am. J. Enol.* **9**, 1.

[4] CHEN, L. F. and LUH, B. S. (1967) *J. Food Sci.* **32**, 66.

[5] GUEFFROY, D. E., KEPNER, R. E., WEBB, A. D. (1971) *Phytochemistry* **10**, 813.

[6] ANDERSON, D. W., GUEFFROY, D. E., WEBB, A. D. and KEPNER, R. E. (1970) *Phytochemistry* **9**, 1579.

[7] FONG, R. A., KEPNER, R. E. and WEBB, A. D. (1971) *Am. J. Enol. Vit.* **22**, 150.

strongly acidic conditions used by Chen and Luh[4] in their investigation would undoubtedly have hydrolyzed any acetylated pigments that were present. For this reason the pigments were extracted from the skins of the Royalty grapes using *n*-pentanol containing 2% formic acid. This mixture extracted acylated pigments fairly free of large amounts of free sugars and polyphenols obtained when methanol is used.[8] Ribéreau-Gayon[9] utilized 0·6% aqueous citric acid for the preparative separation of anthocyanin mono- and di-glucoside pigments into two bands on paper. We found that development of the chromatograms with 1% formic acid gave seven discrete bands, bands *1D* to *5D* were diglucosides and bands *6* and *7* monoglucosides. Approximate R values, relative to the extent of development on the paper, are 0·91, 0·69, 0·62, 0·43, 0·33, 0·17, and 0·09 for bands *1D* to *7*, respectively. Separation of the mono- and di-glucoside bands was enhanced if the chromatographic chamber was saturated with a *n*-pentanol atmosphere. Bands *1D* to *5D* were all highly flourescent whereas bands *6* and *7* were non-fluorescent.

TABLE 1. ROYALTY ANTHOCYANIN PIGMENTS FROM ANALYTIC TLC

Pigment spot*	Identity	R_fs (× 100) BfFW (1st Solv)	PAW (2nd Solv)
I	Malvidin-3-Glc *p*-coumarate	58	73
Ia	Peonidin-3-Glc *p*-coumarate	62	75
IIa	Malvidin-3-Glc acetate	51	63
IIb	Delphinidin-, petunidin-, Cyanidin-3-Glc *p*-coumarates	57	57
IIc	Delphinidin-, petunidin-, Cyanidin-3-Glc acetates	44	53
III	Peonidin-3-Glc	42	58
IV	Malvidin-3-Glc	28	55
1D	Malvidin-, and peonidin-3,5-diGlc acetates	24	52
2D	Malvidin-3,5-diGlc	13	46
3D	Peonidin-3,5-diGlc	17	48
4D	Malvidin- and peonidin-3,5-diGlc *p*-coumarates	29	58
5D	Malvidin- and peonidin-3,5-diGlc caffeoates	30	38

* Three trace spots in the diglucoside region were not investigated.

The analytical separation of the Royalty pigment mixture by TLC was best obtained using a 1 : 1 mixture of silica gel G and cellulose MN 300 developed with a mixture of *n*-butyl formate–formic acid–water (BfFW) in the first direction and *n*-pentanol–acetic acid–water (PAW) in the second direction. Fifteen distinct pigment spots were observed (Table 1). These solvent systems did not cause appreciable hydrolysis of the labile pigments present.

Chen and Luh[4] reported the identification of nine pigments and the possible occurrence of four more in Royalty grapes. In the present study most of the components identified by those authors were again identified using standard techniques.[10] Their identities are indicated in Table 1. Several additional pigments, in particular those acylated with acetic acid, are also indicated in Table. 1.

Band *1D*, the fastest moving band in the preparative separations, was present in the lowest concentration of the diglucoside bands. After the identities of the diglucoside

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[8] ANDERSON, D. W., JULIAN, E. A., KEPNER, R. E. and WEBB, A. D. (1970) *Phytochemistry* **9**, 1569.

[9] RIBÉREAU-GAYON, P. (1959) *Recherches sus Les Anthocyannes Des Vegetaux-Application Au Genre Vitis*, Librarie Generale de l'Enseignement, Paris.

[10] ABE, Y. and HAYASHI, K. (1956) *Botany Mag. (Tokyo)* **69**, 577.

pigments from spots *2D–5D* had been established (Table 1) it was evident that spot *1D* occupied the same position on the TLC relative to the other acylated and non-acylated diglucoside pigments, as malvidin-3-monoglucoside acetate (IIa, Table 1) occupied relative to the other acylated and non-acylated mono-glucoside pigments. Pigment *1D* was unstable to mineral acids, suggesting that it was acylated with acetic acid. Basic hydrolysis and analysis by TLC with several solvent systems gave two spots with R_f values identical to those of malvidin- and peonidin-3,5-diglucosides (*2D* and *3D*). The hydrolysis products from *1D* gave no evidence for the presence of any cinnamic acid by GLC.[6] A differential UV spectrum of *1D*,[8] with a solution of malvidin-3,5-diglucoside in the reference beam, showed essentially no absorption in the region where hydroxycinnamic acids absorb strongly. The IR spectrum of the pigment showed carbonyl absorption at 1720 cm^{-1}, similar to those found for acetylated glucoses and acetylated anthocyanin-3-monoglucosides.[6] No absorptions at 1690, 1600 or 830 cm^{-1} characteristic of *p*-hydroxycinnamic acid derivations of anthocyanin pigments[11] were observed. The NMR spectrum of carefully purified *1D* showed a definite peak at 2·05 ppm indicative of acetate protons; no other protons on the anthocyanin molecule appear in this part of the NMR spectrum. Throughout this study the isolation, separation, and purification solvents used formic acid rather than acetic acid to avoid the possibility that the acetate in the pigment could be an artifact.[12] All evidence obtained is consistent with pigment *1D* consisting of a mixture of malvidin- and peonidin-3,5-diglucosides acylated with acetic acid. Due to its extremely labile nature no attempt has been made to establish the exact position of the acetate moiety. The isolation of pigments acylated with acetic acid from complex pigment mixtures is greatly facilitated by the finding that these pigments will be the fastest moving when the chromatogram is developed with 1% formic acid.

TABLE 2. GLC DATA FOR SILANIZED *p*-COUMAROYLGLUCOSES

Trimethylsilyl derivatives of	Relative retention times*
p-Terphenyl (unsilanized)	1·00
4-*p*-Coumaroyl-D-glucose	2·50, 2·79
6-*p*-Coumaroyl-D-glucose	3·52, 3·69
Residue from pigment oxidation	3·52, 3·71

* Relative to *p*-terphenyl as an internal standard: 3·7 m × 3 mm column, 0·3% OV17, 260°, N_2 40 ml/min.

Pigment *4D*, consisting mainly of malvidin-3,5-diglucoside *p*-coumarate with a small amount of peonidin-3,5-diglucoside *p*-coumarate, was utilized to investigate the position of attachment of the *p*-coumaric acid to the diglucosides. Oxidation with hydrogen peroxide followed by mild basic hydrolysis[13–15] cleaved a *p*-coumaroyl glucose moiety. This glucose was silanized and compared with the silanized derivatives of synthetic 4- and 6-*p*-coumaroyl-D-glucose by GLC[5] (see Table 2). The silanized *p*-coumaroyl-D-glucose isolated from the pigment gave two peaks, representing the anomeric forms, with retention times identical to those of silanized synthetic 6-*p*-coumaroyl-D-glucose. Further

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[11] KOEPPEN, B. H. and BASSON, D. S. (1966) *Phytochemistry* **5**, 183.

[12] TIMBERLAKE, C. F., BRIDLE, P. and TANCHEV, S. S. (1971) *Phytochemistry* **10**, 165.

[13] CHANDLER, B. V. and HARPER, K. A. (1961) *Australian J. Chem.* **14**, 586.

[14] HARBORNE, J. B. (1964) *Phytochemistry* **3**, 151.

[15] TAKEDA, K. and HAYASHI, K. (1964) *Proc. Japan. Acad.* **40**, 510.

confirmation of the position of acylation was obtained using NMR. The isolated *p*-coumaroylglucose was completely acetylated and the acetate proton region of the NMR spectrum was compared with those of the synthetic pentacetyl 4- and 6-*p*-coumaroylglucoses. Previous work had established that acetate proton absorptions at 2·00 and 1·96 ppm resulted from the acetate groups at C_4 and C_6 on glucose respectively.[5] The NMR spectrum of the acetylated *p*-coumaroylglucose isolated from pigment *4D* showed no peak at 1·96 ppm and was identical to the spectrum of the 6-*O*-*p*-acetoxycinnamoyl-1,2,3,4-tetra-*O*-acetyl-*β*-D-glucopyranose.[5]

The GLC and NMR data thus conclusively demonstrate that the malvidin-3,5-diglucoside coumarate isolated from Royalty grapes is acylated in the 6-position of the glucose moiety attached at the 3-position of the malvidin. Pigment *4D* was observed to have an E_{acid}/E_{max} ratio of 67% indicative of only one *p*-coumaric acid group in the molecule.[16]

EXPERIMENTAL

Plant material. The fruit was harvested during the summers of 1969 and 1970 from specimen vines of the hybrid grape variety Royalty in the Foundation Vineyards, University of California, Davis. The sample material was placed in polyethylene bags and stored at −20°.

Isolation of mono- and di-glucoside anthocyanin pigments. ca. 500 g of frozen detached grapes were washed with mixed hexanes (2 × 200 ml) to remove surface wax. The skins were removed, partially dried with filter paper, frozen under liq. N_2 and powdered. The pigments were extracted with 3 × 2% HCOOH in *n*-pentanol (5 : 1, v/w) for 8 hr with vigorous stirring. H_2O (1·5 v) was added to the extracts to form a low boiling azeotrope with water (55% H_2O–45% *n*-pentanol) and the solution evaporated to dryness (30°). The solid residue was redissolved in MeOH (1 : 1, v/g original) and the sol. extracted 5× with mixed hexanes to remove remaining waxes and chlorophyll. The MeOH extracts were combined and the solvent removed under vacuum at 25°. The dried pigment was redissolved in MeOH, chilled to 0°, and centrifuged to remove any insoluble impurities. The soln was then stored at −20°.

Chromatographic separation of anthocyanin pigments. TLC was on well-dried 0·25 mm plates (from 10 g silica gel G plus 10 g of cellullose MN300 in 85 ml H_2O). Solvents were *n*-Bu formate–HCOOH–H_2O (7:2:1) (solvent 1) and fresh *n*-pentanol–HOAc–H_2O (2 : 1 : 1) (solvent 2). The interior of the chromatographic tanks was lined with filter paper saturated with the developing solvent. Preparative PC was on Whatman No. 3MM (previously washed with MeOH) with 1% HCOOH for 11 hr in cabinets saturated with *n*-pentanol. The individual bands were further purified by rechromatography on Whatman No. 3MM paper with *n*-pentanol–HCOOH–H_2O (2 : 2 : 1) for 24 hr. Final purification, when necessary, was by rechromatography with BuOH–HCOOH–H_2O (4 : 2 : 2) and 15% HCOOH. The individual pigments were dissolved in minimum amount of MeOH, precipitated with a 3 × vol Et_2O and the pigment collected by filtration through glass wool. The process was repeated many times until the compound was free of solvents and HCOOH and was subsequently stored under N_2 at −20°.

Spectral measurements. Prior to spectral analysis, the anthocyanin diglucosides were converted into the chloride salt form by soln in minimum anhyd. MeOH, cooled to −30°, and adding anhyd. methanolic HCl (0·1%) until a bright red color was obtained. The solvent was immediately removed under reduced pressure and the pigment dried thoroughly under high vacuum at 30°. UV spectra were in 0·01% methanolic HCl. IR spectra were done using KBr. NMR was in MeOH-d_4 with TMS added as internal standard. The acetylated coumaroyl glucoses were dissolved in $CDCl_3$.

Alkaline hydrolysis was in 10% KOH under N_2 in the dark at 20° for 1 hr. The non-acylated diglucosides were analyzed by TLC with solvent 1 or HOAc–HCl–H_2O (25 : 3 : 72).

Hydrogen peroxide oxidation. Pigment *4D* was oxidized by the method of Chandler and Harper.[13] and hydrolyzed with NH_4OH. The residue was chromatographed in BuOH–HOAc–H_2O (4 : 1 : 2). The UV absorbing *p*-coumaroylglucose was eluted with MeOH, silanized, and analyzed by GLC.

Acetylation of p-*coumaroylglucose* was in pyridine containing 10 mg of NaOAc with Ac_2O overnight at 25°.

Gas Chromatographic analysis of p-*coumaroylglucose.* The compounds were treated with trimethylsilylimidazole in dry pyridine and the mixture heated at 60° for 5 min. The derivatives were analyzed on a 3·7 m × 3 mm column containing 0·3% OV17 on 60–80 mesh Chromosorb G AW/DMCS using FID; column oven temperature 260°; injector temperature 325°; N_2 at 40 ml/min.

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[16] Harborne, J. B. (1958) *Biochem. J.* **70**, 22.