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IDENTIFICATION OF ACETIC ACID AS AN ACYLATING AGENT OF ANTHOCYANIN PIGMENTS IN GRAPES

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Abstract—Acetic acid has been identified by i.r., NMR, and mass spectrometry as a major acylating acid in acylated anthocyanin-3-monoglucosides in grapes.

INTRODUCTION

EXTENSIVE investigations of acylated anthocyanin pigments in a variety of plant materials[1-9] have thus far resulted in the identification of a hydroxycinnamic acid as the acylating group in each case, with *p*-coumaric acid being the most common and caffeic and ferulic acids being present to a lesser extent. In a recent investigation[10] of the anthocyanin skin pigments of *Vitis cinerea* Engelman grapes in our laboratory, evidence was obtained for acylated pigments present in major amounts which contained an acid other than a cinnamic acid derivative as the acylating moiety. Identification of this acid moiety was not possible in the *V. cinerea* studies because of insufficient amounts of fruit. This report describes identification of this acylating acid in pigments isolated from *V. vinifera* var. Teroldico.

RESULTS AND DISCUSSION

The Teroldico pigments were isolated and separated in the same manner as described in the *Vitis cinerea* investigations[10] and each pigment band was shown by similar studies to be identical with the comparable *V. cinerea* pigment band. Of the acylated pigment bands (I, II_A, II_B, II_C), band II_A was present in largest amount, a result consistent with an earlier survey of the pigments of *V. vinifera* grape varieties,[11] and was similar to malvidin-3-monoglucoside in total abundance in the Teroldico pigments. The acylating acids (coumaric in band I, coumaric and caffeic in II_B, and the trace amounts of coumaric and caffeic in II_C)

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were further confirmed by gas chromatography of the silylated acids (Table 1). The silylated caffeic acid was extremely susceptible to hydrolysis and could be observed only if the silylation were done in a vial protected from atmospheric moisture with a septum cap. A differential u.v. spectrum,[10] where a methanol solution of malvidin-3-monoglucoside was used in the reference beam, gave essentially no absorption for the band II_A pigment in the region where hydroxy cinnamic acids absorb strongly. The possibility that malic, succinic, or tartaric acid, common acids in grape systems, could be the acylating acid in the band II_A or II_C pigments was ruled out by gas chromatography of their silyl derivatives (Table 1). The hydrolysis products from the band II_A pigment gave no evidence for the acylating acid present by gas chromatography of the silylation products.

TABLE 1. GAS CHROMATOGRAPHIC INVESTIGATION OF ACYL MOIETIES

Trimethylsilyl derivatives of	Relative retention times	
	1·8 m × 3 mm column*	3·7 m × 3 mm column*
α-Glucose	1·00	1·00
p-Coumaric acid	1·67	
Caffeic acid	3·26	
Ferulic acid	3·63	
Succinic acid		0·35
Malic acid		0·37
Tartaric acid		0·59
Teroldico		
Band I	1·6	
Band II_A	—	—
Band II_B	1·65, 3·22	
Band II_C	1·65†, 3·20†	

* 0·3% OV-17 column, 160°.
† Detected in trace amounts only.

The i.r. spectra of pigment from bands I and II_B show absorptions at 1690, 1600, and 830 cm^{-1} characteristic of 4-hydroxy cinnamic acid esters and consistent with the 1690 cm^{-1} absorption reported by Koeppen and Basson[6] to be characteristic of the carbonyl stretch in the *p*-coumaryl derivative of malvidin-3-glucoside isolated from Barlinka grapes. The i.r. spectrum of band II_A pigment shows a carbonyl absorption at 1726 cm^{-1}, similar to carbonyl absorptions found in acetylated glucoses. The band II_A pigment shows no evidence of the conjugated carbonyl absorption at 1690 or the strong absorption at 1600 cm^{-1} characteristic of 4-hydroxy cinnamic acid esters. The i.r. spectrum of malvidin-3-monoglucoside is essentially identical to that of the band II_A pigment with the exception of the carbonyl absorption at 1726 cm^{-1}. The i.r. spectrum of band II_C pigment indicates a mixture exhibiting characteristics of both a non-conjugated carbonyl and a conjugated 4-hydroxy cinnamic acid ester carbonyl, with the non-conjugated carbonyl predominating.

The NMR spectrum of carefully purified band II_A pigment showed a definitive peak at 2·02 ppm indicative of acetate protons. No other protons on the anthocyanin molecule would appear in this portion of the NMR spectrum. For this study completely new isolation, separation, and purification solvent systems containing propionic acid in place of the usual

acetic acid were developed to avoid the possibility that the acetate in the pigment could be an artifact of the isolation and purification process. The grape berries were also thoroughly washed with hexane before isolation of the pigments in order to remove skin waxes which otherwise show up as a large peak at 1·28 ppm on the NMR spectrum. In view of the composition of the band II_A pigment, approximately 80 per cent malvidin and 20 per cent peonidin derivatives, the relative peak areas of the methoxyl protons (4·0 ppm) and the acetate protons on the NMR spectrum are consistent with one acetate group per anthocyanin molecule. A similar study of the band II_C pigment also gave a definitive peak at 2·02 ppm on the NMR spectrum indicating that the major acylating moiety in this pigment is also acetic acid. The NMR spectrum of band I pigment, which is acylated with *p*-coumaric acid, gave no evidence of the characteristic acetate proton peak at 2 ppm.

Although the NMR spectra gave strong evidence that the acylating moiety of the bands II_A and II_C pigments was acetic acid, further confirmation was sought by mass spectral studies of the acid moiety obtained by basic hydrolysis of the band II_A pigment. The pigment used was isolated and purified using the solvent systems containing propionic acid in place of acetic acid. Removal of the propionic acid from the purified pigment under reduced pressure was extremely difficult, and was best accomplished by repeatedly washing the pigment with diethyl ether. The pigment was redissolved in methanol and evaporated to dryness between each ether washing to avoid retaining propionic acid trapped within the solid pigment. The amount of purified band II_A pigment (*ca.* 10 mg) available for this analysis would give less than 1·5 mg of acetic acid from the basic hydrolysis. Trial runs were made with amounts of glucose pentaacetate sufficient to give several times this amount of acetic acid and the dried hydrolysis product acidified with a trace of concentrated sulfuric acid. All attempts to vacuum transfer the acetic acid from the concentrated sulfuric acid were completely unsuccessful. A system was subsequently developed by which the dried hydrolysis products were acidified with liquid anhydrous hydrogen chloride which could subsequently be separated from the acetic acid at low temperature and pressure. Exhaustive pumping of the dried hydrolysis products from the pigment did not completely remove all traces of methanol, the solvent for the hydrolysis reaction. This resulted in partial conversion of any organic acids present to the corresponding methyl esters under the conditions of the acidification with liquid hydrogen chloride.

The mass spectrum of the volatile material from the hydrolysis of the band II_A pigment was fairly complicated, particularly at *m/e* values below 38, where evidence of traces of methanol, hydrogen chloride, carbon dioxide, and water in the volatile material was clearly apparent. Of most significance in this study was the large peak at *m/e* 43, which is the base peak for both acetic acid and methyl acetate, and is a measure of the total amount of these substances in the volatile material. Acetic acid is also characterized by significant peaks at *m/e* 60, 45, 42, 29, 15, and 14 and methyl acetate by peaks at *m/e* 74, 59, 42, 29, 15, and 14, all of which are present in the spectrum of the pigment volatiles. Evidence was also present in the spectrum to indicate the presence of a trace of propionic acid, from the chromatographic solvent, and a trace of methyl propionate. Because of the complexity of the mixture and the fact that most peaks were characteristic of more than one component, it was not possible to establish the relative ratios of the components in the mixture. In spite of the impossibility of isolating the small amount of acylating acid in pure form, the mass spectral evidence clearly confirms acetic acid as the acylating acid in the band II_A pigment.

The identification of acetic acid as the main acylating moiety in pigment bands II_A and II_C is established by the u.v., i.r., NMR and mass spectral data obtained in this investigation.

It is consistent with the greater observed water solubility of the II_A and II_C pigments relative to the bands I and II_B pigments.[10] Albach *et al.*[3] and Somers[5] in investigating the pigments from *V. vinifera* varieties Tinta Pinheira and Shiraz, respectively, found that the second of the two major acylated pigment bands, separated by means of the butanol–acetic acid–water (BAW) solvent system, was extremely labile. Chromatographic examination of the Tinta Pinheira skin pigments, using the techniques described in the present investigation, has demonstrated the presence of the pigments of bands II_A and II_C in this variety. When chromatographed with BAW these two anthocyanin acetate bands are both part of the labile acylated anthocyanin bands observed by Albach *et al.*[3] and Somers.[5] The instability of these bands is thus explained by the facile hydrolysis of the anthocyanin acetates upon exposure to traces of mineral acids at any time during the isolation, separation, or purification procedures. In almost all investigations of acylated anthocyanins to date, hydrochloric acid has been used at some stage of the study. As a consequence of the instability of anthocyanin acetates to hydrochloric acid, plant materials in which anthocyanin pigments have been characterized with the use of hydrochloric acid need to be re-examined in order to determine whether acetate derivatives occur in genera other than *Vitis*.

EXPERIMENTAL

Plant Material

Fruit from the *Vitis vinifera* var. Teroldico specimen vines in the experimental vineyards, University of California, Davis, was harvested, placed in polyethylene bags, and kept frozen until used.

Isolation of Acylated Anthocyanin Pigments

The extraction and chromatographic separation of the pigments were done in a manner analogous to that described previously for investigation of the acylated anthocyanin pigments in *V. cinerea* grapes[10] with the exception that propionic acid was substituted for acetic acid in all solvent systems, as indicated in the following. The initial extraction of the pigments from the ground grape skins was done with *n*-pentanol–propionic acid (20:1, v/v) and the chromatograms were developed with a $CHCl_3$–propionic acid–H_2O (3:1:2, v/v) (CPW) solvent system using an atmosphere-modifying solution of *n*-pentanol–MeOH–H_2O (10:7:5, v/v).[10] The acylated anthocyanin bands (I, II_A, II_B, II_C) were eluted and further purified by rechromatography on Whatman 3 MM paper with 20% *n*-propionic acid in descending fashion. The purified CPW bands were eluted and the pigments washed ten times with Et_2O and reprecipitated from MeOH as previously described to remove the propionic acid.

Gas Chromatographic Investigation of Acylating Acids

The acids obtained by alkaline hydrolysis of 6–8 mg of each of the purified acylated pigment bands were isolated by Et_2O extraction as previously described,[10] and the Et_2O solution evaporated to dryness in a Microflex tube (Kontes Glass Co., Vineland, N.J.) which was then closed with a Teflon-faced rubber septum and a screw-cap. The acid residue was silylated by the introduction of 20 μl of a solution of trimethylsilylimidazole in dry pyridine (TRI-SIL "Z", Pierce Chemical Co., Rockford, Illinois) through the septum cap with a syringe. The silylated acids, after addition of silylated α-D glucose as an internal standard, were injected onto a 1·8 m × 3 mm OV-17 column, 0·3% on 60/80 mesh Chromosorb G AW/DMCS, in an Aerograph HY-Fi Model 600 D gas chromatograph with flame ionization detection; column oven temp. 160°; injector temp. 275°; nitrogen carrier gas at 30 ml/min. The relative retention times of silylated D,L-tartaric, D,L-malic, and succinic acids to silylated α-D glucose were determined on a 3·7 m × 3 mm OV-17 column using the conditions given above.

Spectral Measurements

U.v. Normal and differential (using a methanol solution of malvidin-3-glucoside in the reference beam) spectra were determined as previously described.[10]

I.r. 1 mg of dried pigment was used to prepare a micro KBr pellet and the spectra determined in a Beckman IR-8 spectrophotometer.

NMR. The purified pigment (6–10 mg) was converted to the Cl-ide form by dissolving in a 0·1% solution of DCl in d_4 methanol and immediately evaporated to dryness on a rotary-film evaporator. The pigment was

redissolved in d_4 methanol, tetramethyl silane added as an internal standard, and the spectra determined on either a Varian HA-100 or A-60 A spectrometer.

Mass. 10 mg of purified washed pigment from Band II_A was hydrolyzed with 0·1 ml of 4% methanolic KOH in the dark for 1 hr at room temp. The methanol was removed by rotary evaporation for 10 min at 5 mm pressure and 30° and finally by evacuation at 10^{-6} torr for 12 hr at room temp. The contents of the reaction flask were then acidified with anhydrous HCl using a vacuum manifold with three standard taper connections as follows. A special test-tube trap, with a 5 cm column of Drierite supported on a glass-wool plug 3 cm above the bottom of the trap, was constructed for the anhydrous HCl. About 1 g of solid HCl was collected in the bottom of the trap by submerging the bottom 3 cm of the trap in liquid N_2 while a slow stream of HCl gas from a cylinder was passed into the trap through a glass capillary inserted through the column of Drierite. The trap was connected to the reaction flask through the vacuum manifold and the anhydrous HCl transferred to the reaction flask by warming the HCl trap to $-83°$ in a dry-ice–acetone bath while the reaction flask was cooled in liquid N_2. After 10 min the dry-ice–acetone and liquid N_2 baths were reversed allowing the HCl to transfer back to the original trap, which was then removed from the system. The reaction flask was evacuated to 1 mm pressure while cooled in the dry-ice–acetone bath. In order to remove as much HCl and CO_2 as possible with a minimum loss of acetic acid, the reaction flask was warmed to $-60°$ with a dry-ice–ortho xylene–meta xylene mixture[12] and evacuated for 5 min at 10^{-6} torr. The acetic acid was then vacuum transferred from the reaction flask (warmed in a water bath at 50°) to a mass spectrometer sample flask cooled in liquid N_2. The material in the sample flask was warmed to $-60°$ and pumped at 10^{-6} torr for an additional 5 min to remove remaining traces of HCl and CO_2. The remaining material was then analyzed with a CEC 21–104 Mass Spectrometer equipped with a digital readout system.

A 10 mg sample of Band I pigment was prepared for mass spectrometric analysis by the same procedure as described above.

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