Elucidation of the Structure and Conformation of Red Radish (*Raphanus sativus*) Anthocyanins Using One- and Two-Dimensional Nuclear Magnetic Resonance Techniques

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Different one- and two-dimensional NMR techniques were used to elucidate the structural conformation of purified anthocyanins obtained from red radish (*Raphanus sativus*). Two novel diacylated anthocyanins, pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-*β*-glucopyranoside] 5-*O*-(6-*O*-malonyl-*β*-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-*β*-glucopyranoside] 5-*O*-(6-*O*-malonyl-*β*-glucopyranoside), were characterized. Two other monoacylated anthocyanins were determined to be pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-*β*-D-glucopyranoside] 5-*O*-(*β*-glucopyranoside) and pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-*β*-glucopyranoside] 5-*O*-(*β*-glucopyranoside). Three-dimensional conformation of the molecule was investigated using NOESY techniques, which showed proximity between hydrogens from the cinnamic acid acylating group and the C-4 of the pelargonidin.

Keywords: *Anthocyanins; red radish (Raphanus sativus L.); 1D and 2D NMR; pelargonidin derivatives*

INTRODUCTION

Interest in anthocyanins has increased in recent years because of their potential use as natural alternatives to artificial colorants. New subfamilies of anthocyanins have emerged over the past decades including the zwitterionic and the polyacylated anthocyanins (Dangles et al., 1993). The zwitterionic anthocyanins are characterized by the presence of the positively charged aglycon and the negative charge from the presence of an aliphatic dicarboxylic acid as acylating group. At the same time, many polyacylated anthocyanins have been found over the last years, most of them having two or more cinnamic acids as acylating groups and also exhibiting increased stability (Rommel et al., 1992; Shi et al., 1992; Baublis et al., 1994; Baublis and Berber-Jiménez, 1995; Giusti and Wrolstad, 1996a,b). In addition, anthocyanins with both types of acyl groups (aliphatic and cinnamic acids) have been found.

Radish anthocyanin pigments were first characterized in the 1960s by different researchers (Ishikura and Hayashi, 1962, 1965; Harborne, 1963; Fuleki, 1969), who determined the presence of pelargonidin 3-sophoroside 5-glucoside (pg-3-soph-5-glu) derivatives, with cinnamic acids attached to the glycosidic moieties. Giusti and Wrolstad (1996a) confirmed the presence of this basic structure in red radishes and found the presence of malonic acid as an additional acylating group. Those findings would place the main anthocyanins present in radishes into both categories mentioned, first with a zwitterionic characteristic given by the charges from the aglycon and the dicarboxylic acid acylation (malonic acid) and second with more than one acylating group, malonic acid and a cinnamic acid group, ferulic or *p*-coumaric acids. Giusti and Wrolstad (1996b) also investigated the potential of red radish anthocyanin extract as a natural colorant for maraschino cherries and determined that radish extract could provide color characteristics very close to those of FD&C Red No. 40 and keep good color and pigment stability for at least 6 months at room temperature. The high stability was attributed to the presence of the acylating groups.

There is a wide variety of chemical structures that can be encountered among anthocyanins: different glycosylating patterns, different acylating groups, many different hydroxyl groups available for esterification of the acylating groups, and finally the presence of cinnamic acids in different stereoisomeric forms (Dangles et al., 1993). In the case of radish anthocyanins, the components of the molecule have been determined but no information was available regarding the position of the acylating groups, the type of cinnamic acid stereoisomer present, or the self-protecting effect of potential interactions of intramolecular copigmentation. The objectives of this study were to determine the positions of the acylating groups, the three-dimensional configuration of radish anthocyanins, and the intramolecular copigmentation of the acylated pigments by one- and two-dimensional nuclear magnetic resonance (NMR) techniques. These NMR techniques were also used to confirm previous identification of the diglucoside as a sophorose unit and to determine the anomeric configuration of the sugars, the types of chemical bonds, and the stereoisomeric conformation of the acylating cinnamic acids.

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MATERIALS AND METHODS

Pigment Extraction. Red radishes cultivar Fuego were grown at the Oregon State University Lewis-Brown Horticultural Farm, Corvallis, OR. Pigment extraction was done following the procedure described by Giusti and Wrolstad (1996b). Radishes were manually peeled, and the frozen epidermal tissue was liquid nitrogen powdered using a stainless steel Waring blendor. Powdered samples were blended with 1 volume of acetone and filtered on a Buchner funnel using Whatman #1 paper. The filter cake residue was reextracted with aqueous acetone (30:70 v/v) until a clear solution was obtained. Filtrates were combined, shaken in a separatory funnel with 2 volumes of chloroform (1:2 acetone−chloroform v/v), and stored overnight at 1 °C. The aqueous portion (top layer) was collected and placed on a Büchi rotovapor at 40 °C until all residual acetone was evaporated (5−10 min). The aqueous extract was made up to a known volume with distilled water.

Anthocyanin Purification. The aqueous extract was passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (Hong and Wrolstad, 1990). Anthocyanins (and other phenolics) were adsorbed onto the minicolumn; sugars, acids, and other water-soluble compounds were removed with 2 volumes of 0.01% aqueous HCl, and anthocyanins were subsequently eluted with methanol containing 0.01% HCl (v/v). The methanolic extract was then concentrated using a Büchi rotovapor at 35 °C, and pigments were dissolved in distilled deionized water containing 0.01% HCl.

Pigment Isolation. Semipurified pigments were isolated using semipreparative HPLC. Individual anthocyanins were collected and further purified by passing them through a C-18 Sep-Pak cartridge. Pigments were recovered from the cartridge with 90% methanol and 10% acidified methanol (0.01% HCl methanol). The methanol was evaporated in a Büchi rotovapor at 35 °C and pure methanol added and evaporated again to favor the removal of HCl or water remaining in the sample. This procedure was repeated twice and a third time using methanol-*d* (CD_3OD). The flask containing dried pure anthocyanins was cooled in a desiccator and the weight recorded. Pure samples were dissolved in methanol-*d* containing 10% TFA-*d* (CF_3COOD).

Alkaline Hydrolysis of Anthocyanins. Approximately 30 mg of purified pigment was hydrolyzed (saponified) in a screw-cap test tube with 10 mL of 10% aqueous KOH for 8 min at room temperature in the dark, as described by Hong and Wrolstad (1990). The solution was neutralized using 2 N HCl, and the hydrolysate was purified using semipreparative HPLC and C-18 Sep-Pak cartridge, as previously described.

High-Performance Liquid Chromatograph (HPLC). *Apparatus.* A semipreparative Dynamax Rainin model SD-300 high-performance liquid chromatograph was used, equipped with a Hewlett-Packard 1040A photodiode array detector and a Gateway 2000 P5-90 computer with Hewlett-Packard HPLC^{2D} ChemStation software. A 1-mL injection loop was used.

Columns and Mobile Phase. A Microsorb C-18 reverse-phase column (5 μm), 250 × 21.4-mm i.d. fitted with a 50 × 21.4-mm i.d. guard module (both from Rainin Instrument Co., Inc., Emeryville, CA) was used. The solvents used were (A) 100% HPLC grade acetonitrile and (B) 1% phosphoric acid (concentrated), 10% acetic acid (glacial), 5% acetonitrile (v/v/v) in water. Flow rate: 20 mL/min. Solvents and samples were filtered through a 0.45-μL Millipore filter type HA (Millipore Corp., Bedford, MA).

HPLC Conditions for Anthocyanin Separation and Isolation. Radish anthocyanins and saponified radish anthocyanins were separated using isocratic conditions at 10% A. The identity of anthocyanins was verified by collecting data at 280, 310, and 520 nm and collecting peak spectra (from 260 to 600 nm) of all peaks at 520 nm. A total of 15, 25, 50, 60, and 55 mg of pigments 1−5 were collected.

Electrospray Mass Spectroscopy. Low-resolution MS was done using electrospray MS. The instrument was a Perkin-Elmer SCIEX API III+ mass spectrometer, equipped with an ion spray source (ISV = 4700, orifice voltage of 80) and loop injection. Purified anthocyanins were injected directly into the system.

Nuclear Magnetic Resonance (NMR). *Apparatus.* One-dimensional (1D) and two-dimensional (2D) NMR spectra were collected on a Bruker 600 operating system at 14.1 T with an inverse probe.

Conditions for Analyses. Samples analyzed were dissolved in MeOH-*d* (CD_3OD) containing 10% TFA-*d* (CF_3COOD), and all analyses were carried out at 25 °C.

Hydrogen NMR. 1D ^{1}H NMR was recorded using a spectral width of 9 ppm, with center at 5 ppm. Two-dimensional correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were collected using the average of 32 transients for each of the 256 increments in t_1, with 4096 complex points in t_2. A spectral width of 10 ppm with center at 5 ppm was used. Spin coupling information was obtained using homonuclear *J*-resolved spectroscopy, with a spectral width of 10 ppm and center at 5 ppm.

Carbon NMR. The proton-detected ^{13}C heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra were obtained using spectral widths of 9 ppm with center at 5 ppm in the proton dimension and of 200 ppm with center at 100 ppm in the carbon dimensions. A total of 32 transients were averaged for each of the 512 increments in t_1, with 2048 complex points in t_2. The (1/2*J*) delays in the pulse sequence were optimized for proton−carbon coupling constants of approximately 160 Hz.

Nuclear Overhauser Effect Spectroscopy (NOESY). Spectra were collected using the average of 64 transients for each of the 256 increments in t_1, with 4096 complex points in t_2. The spectral width used was 10 ppm with center at 5 ppm.

RESULTS AND DISCUSSION

HPLC Analysis and Mass Spectroscopy of Radish Anthocyanins. All acylated and saponified radish anthocyanins were isolated using semipreparative HPLC, and the purity of the isolated pigments ranged from 92% to 99%, as indicated by their percent area at 280 nm. Previous chemical characterization of radish anthocyanins (Giusti and Wrolstad, 1996a) revealed the presence of four major anthocyanins, all sharing a basic structure: pelargonidin aglycon with a diglucoside on position 3, identified as sophorose-based on reported identification of the pigments (Ishikura and Hayashi, 1963; Fuleki, 1969; Harborne, 1963), and a glucose unit on position 5 of the anthocyanidin structure. Two of those pigments (ca. 20% of total pigment content) were monoacylated with *p*-coumaric (pigment 2) or ferulic (pigment 3) acids, and two others (ca. 70% of total pigment content) were diacylated anthocyanins, containing the same cinnamic acid acylations plus malonic acid (pigments 4 and 5, respectively). The remaining anthocyanin content (ca. 10%) was composed of four other minor peaks with the same basic structure but with either no acylation, monoacylation with caffeic acid, or diacylation with cinnamic acids.

Electrospray mass spectroscopy confirmed previously reported *m*/*z* of the molecular ions as 903, 933, 989, and 1019 for pigments 2, 3, 4, and 5, respectively (Giusti and Wrolstad, 1996a). Saponification of the pigments yielded only one pigment (pigment 1), pelargonidin triglucoside, with a *m*/*z* ratio of 721.

Purified nonacylated raphanusin did not bind to the C-18 resin as easily and tightly as other anthocyanins did, due to its increased polarity and high hydrophilicity caused by the presence of three sugar substituents. This increased hydrophilicity also accounted for a low affinity and very short retention time on the C-18 HPLC column. Several semipreparative HPLC runs were

Table 1. ^{1}H NMR Spectra (Chemical Shifts, ppm) of Radish Anthocyanins (in 10% TFA-*d*, MeOH-*d*)

	pigment[a]			
	1	**2**	**4**	**5**
		Aglycon		
pg C-4	9.19	9.03	8.97	8.98
pg C-6	7.15	6.97	6.98	6.96
pg C-8	7.15	7.02	6.97	6.94
pg C-2′	8.66	8.60	8.58	8.57
pg C-3′	7.10	7.10	7.10	7.08
pg C-5′	7.10	7.09	7.10	7.08
pg C-6′	8.66	8.61	8.58	8.57
		Sophorose Glycoside (G1)		
C-1	5.49	5.61	5.61	5.61
C-2	4.12	4.12	4.13	4.13
C-3	3.81	3.84	3.86	3.88
C-4	3.63	3.61	3.63	3.64
C-5	3.50	3.86	3.96	3.97
C-6	3.78	4.46	4.45	4.38
	3.95	4.53	4.54	4.59
		Sophorose Glycosyl (G2)		
C-1	4.85	4.79	4.81	4.82
C-2	3.21	3.25	3.27	3.28
C-3	3.30	3.33	3.36	3.35
C-4	3.26	3.27	3.26	3.28
C-5	3.00	3.08	3.05	3.07
C-6	3.50	3.49	3.52	3.52
	3.60	3.60	3.62	3.60
		Glucoside (G3)		
C-1	5.20	5.19	5.22	5.20
C-2	3.73	3.76	3.80	3.78
C-3	3.60	3.60	3.60	3.57
C-4	3.48	3.49	3.48	3.42
C-5	3.69	3.61	3.82	3.80
C-6	3.77	3.77	4.28	4.21
	3.96	3.99	4.56	4.54
		Acyl Group		
C-2		7.27	7.19	6.85
C-3		6.78	6.72	
C-5		6.78	6.72	6.72
C-6		7.27	7.19	6.82
C-7		7.35	7.35	7.34
C-8		6.16	6.18	6.18
		Malonyl		
C-2			3.45	3.49

[a] 1, pg-3-soph-5-glu; 2, pg-3-soph-5-glu acylated with *p*-coumaric acid; 3, pg-3-soph-5-glu acylated with ferulic acid; 4, pg-3-soph-5-glu acylated with *p*-coumaric acid and malonic acid; 5, pg-3-soph-5-glu acylated with ferulic and malonic acids.

needed to collect amounts large enough to perform the NMR analyses on the nonacylated pigment. Even then, the concentration was relatively low and only a limited number of NMR analyses were performed on this sample; thus, no carbon data was obtained for it.

NMR of Saponified Radish Anthocyanins. Saponification of radish anthocyanin yielded only one pigment (pigment 1) previously identified (Giusti and Wrolstad, 1996a) by HPLC and electrospray mass spectroscopy as pelargonidin 3-sophoroside 5-glucoside. The chemical shifts (δ) obtained from the ^{1}H NMR analysis of saponified radish anthocyanins (Table 1) confirmed the identity of the pigment.

Hydrogens from the aglycon gave chemical shifts that ranged from 7.1 to 9.19 ppm, in agreement with the aromaticity of the A and B rings (Figure 1) of the aglycon molecule and with previously reported data for similar compounds (Hosokawa et al., 1995; Fossen et al., 1996).

Three signals corresponding to the chemical shifts of acetal groups formed by the anomeric carbons from

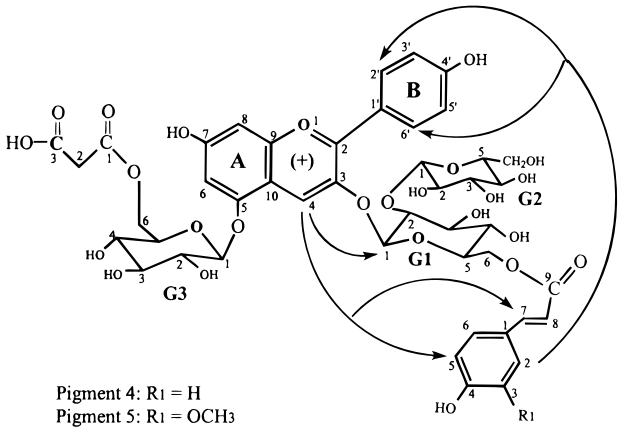

Figure 1. Chemical structure of pelargonidin derivatives encountered in red radish. Arrows indicate hydrogens close in space.

sugar residues were detected and assigned to the different sugars present in the molecule using previously reported data for similar compounds (Terahara et al., 1996; Hosokawa et al., 1995) as reference. The chemical shift of 5.49 ppm was assigned to the proton at the anomeric carbon of the glycoside residue from sophorose (G1, Figure 1), 4.85 ppm assigned to the glycosyl residue from sophorose (G2), and 5.20 ppm assigned to the anomeric proton of the glucoside residue (G3). Important information can be obtained from the H−H coupling constants of neighboring hydrogens. Neighboring diaxial hydrogens can be clearly identified by large coupling constants (7−9 Hz), which contrast with smaller coupling constants (2−4 Hz) of protons in diequatorial or axial−equatorial configurations (Agrawal, 1992; Breitmaier, 1993). This difference allows for elucidation of the configuration of sugars. Clear duplets were observed for the anomeric protons of the saponified radish anthocyanin, and the *J*-couplings for these protons were determined from the ^{1}H NMR spectra. The large coupling constants obtained for hydrogens at the anomeric carbon of the sugar residues (7.1, 7.77, and 7.76 Hz for G1, G2, and G3, respectively) showed that the hydrogens at the anomeric carbon and at carbon 2 of the glucose units were all in an axial configuration, revealing that all glucose units were in the β-configuration.

The vast majority of proton resonances from nonanomeric sugar methine and methylene groups appear in a small spectral width of 3.0−4.2 ppm, with subsequent overlap problem (Agrawal, 1992). Significant overlap makes it difficult to locate the resonance of individual nuclei and to assign these resonances to a specific monosaccharide residue. Two-dimensional shift correlation and total correlation analyses (2D NMR COSY and TOCSY) assisted in assigning all individual chemical shifts of the hydrogens from the different sugars (Table 1), using as a reference the already assigned resonances of the anomeric carbons and correlating spins in a stepwise manner around the spin system of the ring. The higher chemical shift obtained for the proton at position 2 in G1 (4.12 ppm as compared to 3.21 and 3.73 ppm for H-2 in G2 and G3, respectively) showed the presence of a substitution on that position, in agreement with the bond type 1−2 of sophorose.

NMR of Acylated Radish Anthocyanins. The NMR spectra obtained for all radish anthocyanins had similar chemical shifts for the aglycon (pelargonidin) as well as for most protons from the sugar substitutions

Table 2. ^{1}H and ^{13}C NMR Spectra of Pelargonidin 3-Sophoroside 5-Glucoside Acylated with Malonic Acid and *p*-Coumaric (Pigment 4) or Ferulic (Pigment 5) Acid

	pigment 4					pigment 5				
	^{1}H NMR		(*J*, Hz)	HMQC	HMBC	^{1}H NMR		(*J*, Hz)	HMQC	HMBC
					Aglycon					
pg C-2					164					164.2
pg C-3					144.5					144.5
pg C-4	8.97	d	(2.4)	134.7		8.98	d	(3.0)	134.8	
pg C-5					156.5					155.8
pg C-6	6.98	d	(1.56)	105.0		6.96	br s		104.6	
pg C-7					168					169.0
pg C-8	6.97	d	(1.56)	96.3		6.94	br s		96.5	
pg C-9					156.5					155.8
pg C-10					112.5					112.3
pg C-1′					119.5					119.7
pg C-2′	8.58	d	(9.05)	135.5		8.57	d	(8.9)	135.5	
pg C-3′	7.10	d	(9.04)	116.9		7.08	d	(8.89)	117.4	
pg C-4′					166.5					166.7
pg C-5′	7.10	d	(9.04)	116.9		7.08	d	(8.89)	117.4	
pg C-6′	8.58	d	(9.05)	135.5		8.57	d	(8.9)	135.5	
					Sophorose Glycoside (G1)					
C-1	5.61	d	(7.14)	100.5		5.61	d	(6.98)	110.5	
C-2	4.13	dd	(7, 8.5)	80.8		4.13	dd	(6.3, 9.3)	80.5	
C-3	3.86	dd	(8.25, 9.25)	76.8		3.88	dd	(7.2, 9.0)	76.6	
C-4	3.63	dd	(8.5, 10)	70.8		3.64	dd	(8.4, 10.2)	70.5	
C-5	3.96	m		74.5		3.97	m		74.2	
C-6	4.45	dd	(7.65, 12.02)	63.5		4.38	dd	(7.5, 11.7)	63.0	
	4.54	br d	(11.85)			4.59	dd	(2.7, 11.7)		
					Sophorose Glycosyl (G2)					
C-1	4.81	d	(7.77)	103.6		4.82	d	(7.61)	103.0	
C-2	3.27	dd	(7.5, 9.5)	74.7		3.28	dd	(8.1, 9.9)	74.8	
C-3	3.36	dd	(8.75, 9.75)	76.8		3.35	dd	(7.5, 10.5)	76.5	
C-4	3.26	dd	(8.5, 10)	70.7		3.28	dd	(8.1, 9.9)	70.3	
C-5	3.05	m		76.9		3.07	dd	(6.0, 9.6)	77.0	
C-6	3.52	br s		61.6		3.52	dd	(6.3, 12.3)	61.0	
	3.62	dd	(8.5, 10)			3.60	dd	(8.4, 10.2)		
					Glucose (G3)					
C-1	5.22	d	(7.76)	101.5		5.20	d	(7.69)	101.5	
C-2	3.80	dd	(7.75, 9.75)	74.0		3.78	dd	(7.5, 9.3)	73.8	
C-3	3.60	dd	(8.5, 9.5)	76.5		3.57	dd	(8.7, 9.9)	76.6	
C-4	3.48	dd	(8.5, 10)	70.0		3.42	dd	(7.5, 10.5)	69.9	
C-5	3.82	m		74.7		3.80	dd	(6.6, 9.6)	74.5	
C-6	4.28	dd	(6.28, 11.99)	64.2		4.20	dd	(6.6, 11.4)	64.1	
	4.56	br d	(11.85)			4.54	dd	(2.7, 12.3)		
					Cinnamic Acid					
C-1					126					126.5
C-2	7.19	d	(8.53)	130.5		6.85	br s		111.0	
C-3	6.72	d	(8.55)	115.5						148.0
C-4					160.5					149.7
C-5	6.72	d	(8.55)	115.5		6.72	d	(8.16)	115.5	
C-6	7.19	d	(8.53)	130.5		6.82	d	(9.65)	123.0	
C-7	7.35	d	(15.87)	145.8		7.34	d	(15.84)	147.0	
C-8	6.18	d	(15.89)	113.5		6.18	d	(15.85)	114.0	
C-9					168					168.0
					Malonyl					
C-1					167					167.5
C-2	3.45	s		40.5		3.49	s		40.5	
C-3					167					168.0

(Table 1). Differences were found in the shifts of protons at position 6 of G1 (on pigments 2, 4, and 5) and G3 (on pigments 4 and 5), higher than those obtained for H-6 of the sugars in the saponified pigment. This suggested that the acylating groups were attached to the hydroxyl groups of these positions.

Additional signals were detected in all acylated pigments analyzed in the region between 6.1 and 7.4 ppm, corresponding to protons from the cinnamic acid acylating groups. Another additional signal detected on the ^{1}H NMR spectra of pigments 4 and 5 in the 3.4 ppm region was identified as coming from the proton at position 2 of the malonic acid residue. This signal was not evident at first due to its proximity to signals from protons located on the sugar residues and from the solvent (3.3 ppm).

Two-dimensional HMQC and HMBC spectra were collected (Table 2) for pigments 2, 4, and 5 to confirm the position of the ester linkage. HMQC and HMBC experiments enable us to trace connectivities between ^{1}H and ^{13}C atoms through indirect detection of the low-natural abundance nuclei ^{13}C, via ^{1}H nuclei (Agrawal, 1992). The HMQC spectra provides correlation between directly bonded ^{1}H and their corresponding ^{13}C. The ^{13}C NMR spectra for these pigments were obtained using this procedure, and the results are presented in Table 2. HMBC is a long-range heteronuclear chemical shift correlation technique and provides intraresidue multiple

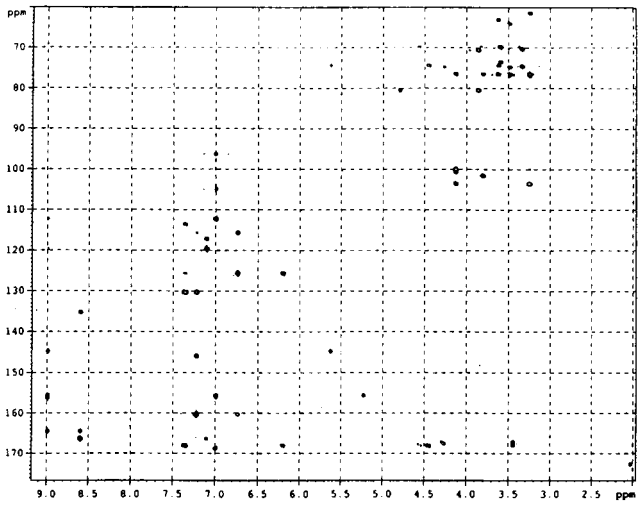

Figure 2. Heteronuclear multiple bond correlation (HMBC) data of pigment 4.

bond correlation; this information is valuable for confirming ^{13}C and/or ^{1}H assignments. At the same time, it also provides interresidue multiple bond correlation between the anomeric carbon and the aglycon proton and thus serves to identify the interglycosidic linkages (Agrawal, 1992). HMQC techniques were useful for confirmation of the signal at 3.4 ppm as coming from malonic acid, since this ^{1}H chemical shift was the only one that correlated with ^{13}C signals of 40.5 ppm, typical for such a structure and in contrast with all the other ^{13}C chemical shifts of these molecules, higher than 60 ppm.

Figure 2 shows the HMBC plot for pigment 4. The HMBC spectra of pigments 2 and 4 showed correlation between the H-6 of G1 (4.45 and 4.54 ppm) and a carbonyl carbon from the *p*-coumaric acid (167.8 ppm), confirming that the *p*-coumaric acid was esterified to sugar G1 through the hydroxyl group at C-6. The HMBC spectra of pigment 4 (Figure 2) also showed correlation between H-6 of G3 (4.28 and 4.56 ppm) and the carbon from the carbonyl group of malonic acid (167.1 ppm). These results showed that *p*-coumaric acid was attached to position 6 of G1 through an ester bond, while malonic acid was attached through an ester bond to position 6 of G3. Similar results were obtained for pigment 5, where the position of the ester linkage between the ferulic acid and sugars was determined to be at C-6 of G1; malonic acid was attached to C-6 of G3. The positions of attachment of the acyl groups were confirmed by the higher chemical shifts obtained for H-6 of G1 and G3 and by the correlation found between these hydrogens and the carboxylic groups of the adjacent acylating acids.

The chemical shift and large *J*-coupling constants (15.89 Hz) of H-7 and H-8 (Table 2) for *p*-coumaric acid (in pigments 2 and 4) and ferulic acids (pigment 5) showed that both cinnamic acids were in the *trans* configuration. Thus, pigment 2 was identified as pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-*β*-glucopyranoside] 5-*O*-(*β*-glucopyranoside), pigment 4 was pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-*β*-glucopyranoside] 5-*O*-(6-*O*-malonyl-*β*-glucopyranoside), and pigment 5 was pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-*β*-glucopyranoside] 5-*O*-(6-*O*-malonyl-*β*-glucopyranoside), where the latter two are novel anthocyanins.

Pigment 3 was not analyzed by NMR. However, the information obtained for pigments 2, 4, and 5, combined with the fact that the degradation of pigments 4 and 5 results in the formation of pigments 2 and 3, respectively, with release of malonic acid (Giusti and Wrolstad, 1996b), suggests the chemical structure of pigment 3 as pelargonidin 3-*O*-[2-*O*-(*β*-glucopyranosyl)-6-*O*-(*trans*-feruloyl)-*β*-glucopyranoside] 5-*O*-(*β*-glucopyranoside).

Copigmentation of Acylated Radish Anthocyanins. Two-dimensional NOESY experiments provide valuable information in mapping specific through-space internuclear distances (Keepers and James, 1984) which could be sufficient to determine the molecular three-dimensional structure. Cross-peaks are observed in NOESY spectra between proton pairs that are close in space, typically less than 5 Å, close enough to allow though-space interactions (Agrawal, 1992), and the greater the signal, the closer together those hydrogens are in space (Kemp, 1991). NOESY experiments performed on anthocyanin pigments 4 and 5 showed high correlation between H-4 of the pelargonidin moiety and the hydrogens in positions H-3 and H-5 of *p*-coumaric acid, as well as a small correlation with H-8. It has been proposed (Goto, 1987; Figueiredo et al., 1996a,b) that the stacking between the aromatic nuclei of the anthocyanin and the planar ring of the aromatic acid would be through the formation of $\pi-\pi$ hydrophobic interactions. Our findings are in agreement with the proposed folding of anthocyanins related to intramolecular copigmentation; however, it is also possible that the through-space interactions were due to intermolecular association.

Proximity between malonic acid and the pyrylium ring of the anthocyanin could not be determined due to the weak signal obtained for hydrogens from malonic acid as well as the interference of the signals of hydrogens from the sugar moieties and the solvent system, very close to those of malonic acid. However, the zwitterionic character of pigments 4 and 5 would likely make an ionic attraction between the positively charged portion of the oxonium form and the negatively charged free carboxylic group from malonic acid. The positions of attachment of the acylating groups to the sugars, at position C-6 in both cases, would allow for free rotation and the subsequent folding of the acyl groups over the pyrylium ring in a sandwich-type conformation, where cinnamic and malonic acids would be on the top and the bottom of the pelargonidin portion of the molecule.

Several studies (Brouillard, 1981; Goto, 1987; Goto and Kondo, 1991; Figueiredo et al., 1996a,b) have suggested that the intramolecular copigmentation or interaction within anthocyanins may play an important role in the increased stability of acylated anthocyanins. The flexible saccharide chains can act as linkers allowing the folding of the acyl aromatic rings over the planar pyrylium ring preventing the addition of nucleophiles, especially water, to the C-2 and C-4 positions of the anthocyanin, protecting the chromophore against hydration and diminishing the formation of the colorless pseudobase (Figueiredo et al., 1996a). Copigmentation has also been reported to have an impact on the anthocyanin spectral characteristics (Mazza and Brouillard, 1990) causing a hyperchromic and bathochromic effect and increasing the color intensity as well as the wavelength of maximum absorbance of the pigment. The possible folding of acylated radish anthocyanins may

account for the high color and pigment stability reported (Giusti and Wrolstad, 1996b) for these pigments in food systems.

CONCLUSIONS

One- and two-dimensional NMR techniques were employed to chemically characterize red radish anthocyanins. Two novel zwitterionic diacylated anthocyanins and two monoacylated pelargonidin derivatives were found and their molecular structures elucidated. Spatial proximity between hydrogens from the cinnamic acid acylating group and the anthocyanin moiety was revealed by NOESY techniques. These correlations suggested copigmentation, which may contribute to the high pigment stability previously reported for these pigments.

ABBREVIATIONS USED

ACN, anthocyanins; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; 1D and 2D NMR, one-dimensional and two-dimensional nuclear magnetic resonance; pg-3-soph-5-glu, pelargonidin 3-sophoroside 5-glucoside, also called raphanusin.

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