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Red cabbage anthocyanins: The influence of D-glucose acylation by hydroxycinnamic acids on their structural transformations in acidic to mildly alkaline conditions and on the resulting color

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ABSTRACT

Anthocyanins acylated by hydroxycinnamic acids (HCAs) are fascinating plant pigments that express a variety of red, purple and blue colors by combining multiple structural transformations and molecular interactions. Acylated anthocyanins are also a promising alternative to artificial food colorants. In this work, the mono- and diacylated anthocyanins of red cabbage have been extensively studied by NMR, UV–visible spectroscopy and circular dichroism. Our results show that HCA residues promote π-stacking interactions between the phenolic nuclei, thereby efficiently protecting the cyanidin chromophore against water addition leading to colorless forms. For instance, the rate constant of water addition is *ca.* 0.3 s^{-1} for the nonacylated pigment, in the range 0.1–0.2 s^{-1} for the three monoacylated pigments and of the order of 0.01 s^{-1} for the three diacylated pigments. By contrast, the rate constant of water elimination and the thermodynamic constants of proton transfer between the cationic, neutral and anionic colored forms are only weakly affected by acylation. Thus, through π-stacking interactions, the diacylated anthocyanins maintain a higher percentage of cationic and neutral colored forms at equilibrium in mildly acidic conditions. In neutral - mildly alkaline conditions, the diacylated anthocyanins adopt persistent anionic forms (very slow water addition), expressing intense blue colors. NMR and CD data suggest that a combination of cyanidin – HCA (intramolecular copigmentation) and cyanidin – cyanidin (self-association) interactions operates in the color-stabilizing mechanism, which is also translated in an improved resistance against the long-term color loss in mildly alkaline conditions, signalling the irreversible degradation of the chromophore.

1. Introduction

Anthocyanins (plant pigments) acylated on their sugar moieties by hydroxycinnamic acid (HCA) residues are known to express more intense and more stable color in mildly acidic to neutral aqueous solution than their nonacylated analogs [1,2]. Depending on the glycosidation site (A-, B- or C-ring), type of sugars (monosaccharides, linear or branched oligosaccharides) and acylation site (primary *vs.* secondary OH groups of sugars), acylated anthocyanins also display the specificity of a) either adopting folded conformations in which the chromophore and HCA residues are in van der Waals contacts (attested by the observation of long-range NOE correlations by ^{1}H NMR), a phenomenon known as intramolecular copigmentation, or b) form noncovalent dimers (possibly, higher oligomers) by chiral stacking of the chromophores, which is strengthened by the acyl residues (attested by the observation of exciton-type bands in the visible range by circular dichroism).

Examples of the remarkable sensitivity of anthocyanin behavior as a function of the acylation site can be found in the literature. For instance, petanin is a monoacylated anthocyanin having a linear disaccharide moiety (1,6 inter-glycosidic linkage) at C3-OH whose terminal sugar is acylated at C4-OH. Its NOESY spectrum suggests the formation of an extended head-to-head dimer with both flavylium nuclei stacked on one another and both HCA residues too [3]. Alatanin C

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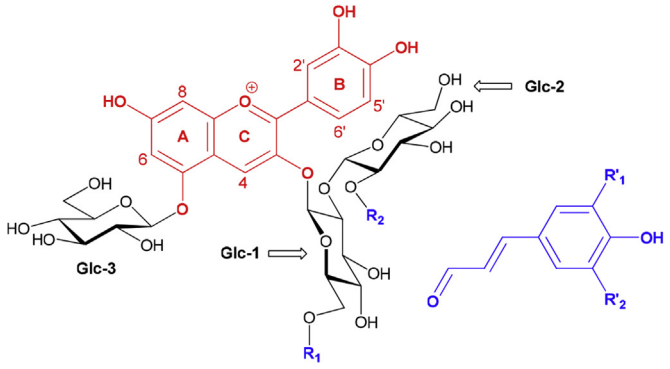

Pigment	R_1	R_2
Pigment A	H	H
P1	pC	H
P2	Fl	H
P3	Sp	H
P4	pC	Sp
P5	Fl	Sp
P6	Sp	Sp
Acyl group	**R'_1**	**R'_2**
pC	H	H
Fl	OMe	H
Sp	OMe	OMe

Scheme 1. Structure of the red cabbage anthocyanins.

is closely related to petanin but probably more flexible as acylation now occurs at C6-OH. Consequently, its NOESY spectrum reveals flavylium-HCA contacts [4]. Finally, tecophilin displays a D-glucose unit acylated by HCA residues at both C2-OH and C6-OH. As expected, only the flexible one (at C6-OH) stacks onto the chromophore [5].

Red cabbage is a very interesting source of acylated anthocyanins [6,7]. As sophisticated anthocyanins expressing a high degree of acylation are generally found in flowers [2], not in fruit and vegetables, red cabbage is even one of the few known dietary sources of diacylated anthocyanins. Red cabbage anthocyanins (RCAs) are cyanidin (3,3′,4′,5,7-pentahydroxyflavylium) derivatives with a *β*-sophorose (D-Glc-β-1,2-D-Glc) moiety at C3-OH and a *β*-D-glucose moiety at C5-OH (Scheme 1). The reference (nonacylated) pigment is named pigment A. Only the sophorose part is acylated and acylation occurs on the primary OH group (C6-OH) of the first Glc (Glc-1) and/or one of the secondary OH group (C2-OH) of the second Glc (Glc-2) [8–10]. Overall, RCAs mainly consist in a series of monoacylated pigments P1-P3 with *p*-coumaric (pC), ferulic (Fl) and sinapic (Sp) acid residues at Glc-1 and the homologous series of diacylated pigments P4-P6 with an additional Sp residue at Glc-2. Each of these seven RCAs (including pigment A) was isolated and quantitatively investigated for its proton transfer and water addition equilibria over a wide pH range from 1 to 8. This systematic study sheds light on the strong influence of acylation by HCAs on the color intensity at equilibrium and on the subtle mechanisms at work in providing color stability. These findings provide important clues to assess the potential of RCAs as food colorants expressing red to blue colors from acidic to mildly alkaline conditions.

2. Experimental section

2.1. Materials

RCAs were first purified from red cabbage extracts by elution on C18 silica cartridges (removal of sugars and organic acids). Then, individual pigments were isolated by semi-preparative high performance liquid chromatography (HPLC) and characterized by analytical HPLC coupled to diode array and mass spectrometry detectors. The corresponding procedures were already reported with details [6,11].

2.2. Nuclear magnetic resonance (NMR)

NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) in a 5-mm diameter tube at variable temperature using 5% CF_3COOD-CD_3OD as a solvent. Chemical shifts were recorded as parts per million (ppm) using the CD_2HOD resonance as a standard (3.31 ppm). Various 1D and 2D measurements were carried out for structure identification and assignment of signals using the previously reported irradiation experiments [5,12].

2.3. UV–visible spectroscopy

Spectra were recorded on an Agilent 8453 diode array spectrometer equipped with a magnetically stirred quartz cell (optical pathlength = 1 cm). The temperature in the cell was controlled by means of a water-thermostated bath at 25.0 ± 0.1 °C.

2.4. Circular dichroism (CD)

Each of the selected pigments (pigment A, P3, P4, P5, P6) was dissolved in 0.1% HCl-MeOH, then diluted in aqueous solutions at pH = 1.0 and pH = 8.0 to a final concentration of 50 μM. Each solution was transferred into a cuvette of 1 cm cell-length and CD spectra were recorded on a JASCO J-720 spectrometer.

2.5. Kinetics of water addition

Aqueous buffer solutions of 0.01 M sodium citrate/HCl (pH 2–5) and 0.01 M Na_2HPO_4/NaH_2PO_4 (pH 5–8) were prepared in 0.1 M KCl (ionic strength buffer). Concentrated stock solutions of RCAs were diluted to *ca.* 5 mM in 0.1 M aqueous HCl (pure flavylium form). Two ml of the buffer solution were placed into the spectrometer cell and 20 μL of the anthocyanin solution were rapidly added and the spectra recorded at regular time intervals (from 0.5 to a few seconds depending on pH and the apparent rate of water addition). Once equilibrium is reached (from *ca.* 10 s to a few min depending on pH), the pH of each solution was measured.

Alternatively, the kinetics of water addition can be investigated by a pH jump into RCA solutions in 0.01 M sodium citrate - HCl (pH ≈ 1.8). Aliquots (2 mL) of these solutions were placed into the spectrometer cell and a small volume (20–80 μL) of 1 M aqueous NaOH was added (final pH up to 5).

Whatever the method used, the apparent first-order rate constant (k_{obs}), initial and final absorbance values (A_0, A_f) at the flavylium's λ_{max} were collected. From the pH dependence of k_{obs} and A_f/A_0, the thermodynamic constant for the first proton transfer (pK_{a1}) and the rate constants of hydration (k_h) and dehydration (k_{-h}) were estimated.

2.6. Thermodynamics of water addition and second proton transfer

After the kinetic experiments, the equilibrated solutions were collected and their UV–visible spectra recorded. From the plot of A_{eq} (at the flavylium's λ_{max}) as a function of pH in the range 2–5, the overall acidity constant pK'_a and apparent hydration constant pK'_h ($K'_a = K'_h + K'_{a1}$) were estimated. In the pH range 6–8, water addition