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Impact of a pectic polysaccharide on oenin copigmentation mechanism

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ABSTRACT

Copigmentation plays an important role in the colors provided by anthocyanins. However, little attention has been paid to the interaction between anthocyanins and cell wall compounds (e.g. polysaccharides) and the impact of this interaction on anthocyanins color, a fundamental issue to be considered in industrial applications of these pigments as food colorants. The copigmentation binding constants (K_{CP}) for the interaction between malvidin-3-*O*-glucoside and (+)-catechin in the presence of low methoxylated pectic polysaccharide were determined. The values obtained showed that in the presence of pectic polysaccharide the copigmentation binding constants decreased. These results probably suggest the occurrence of competition equilibrium in which the presence of pectin limited the association between catechin and oenin. ^{1}H NMR studies revealed that the dissociation constant determined for these complexes was very similar in absence and presence of 1.5 g/L pectin with this polysaccharide apparently not affecting the strength of anthocyanin-catechin binding.

1. Introduction

Anthocyanins are plant pigments widespread in the Nature responsible for a great diversity of colors of many flowers, fruits and beverages, particularly red wine (González-Manzano, Santos-Buelga, Dueñas, Rivas-Gonzalo, & Escribano-Bailón, 2008). However, the color displayed by these pigments is structurally dependent on the conditions and composition of the media where they are dissolved. On the other hand anthocyanins may suffer interactions among them and with other compounds that influence their chemical equilibria and modify their color (Santos-Buelga, Mateus, & De Freitas, 2014). At pH lower than 2, the red flavylium cation (AH^+) is predominant. As pH increases up to pH values of 3–4, the flavylium cation is involved in two parallel reactions: (i) deprotonation to form the blue-purple quinoidal bases (A); (ii) hydration in position C_2 or C_4 followed by proton loss to give the colorless hemiketal (B), which is in fast equilibrium with the yellow *cis*-chalcone (C*c*). Finally, the *cis-trans* isomerization leads to a small mole fraction of *trans*-chalcone (C*t*) (Brouillard & Dubois, 1977). Despite the thermodynamic tendency of anthocyanins to form colorless hemiketals, in their natural media these pigments are able to express intense and stable colors. This evidences naturally occurring stabilization mechanisms, improving chemical and colorimetric stability of anthocyanins a fundamental aspect to be considered in the technological application of these pigments. Copigmentation is regarded as a significant factor on anthocyanins coloration playing a major role in the expression of a wide range of colors provided by these pigments (Boulton, 2001). Copigmentation refers to non-covalent interactions between the planar polarizable nuclei of anthocyanins colored forms (both flavylium cation and quinoidal bases) and colorless organic molecules (copigments) forming molecular associations or complexes. This association is promoted by weak molecular forces, including hydrogen-bind interaction and hydrophobic effects, mainly dispersive π–π stacking interactions between the polarizable orbitals of the aromatic rings (Asen, Stewart, & Norris, 1972). Copigmentation complexes adopt a sandwich configuration (vertically stacked) involving π–π interactions or more perpendicular alignments based on CH-π interaction (Levengood & Boulton, 2004) physically limiting water access to the chromophore, thereby preventing at least in part the formation of anthocyanin colorless forms (limiting the occurrence of the hydration reaction). Usually, copigmentation results in enhanced absorption (hyperchromic effect) and may also result in a shift in the wavelength maximum to shorter (hypsochromic shift) or longer wavelength (bathochromic effect) (Brouillard, Chassaing, & Fougerousse, 2003). The usual copigments include a variety of compounds such as phenolic acids, flavan-3-ols, flavonols, amino acids, alkaloids and polysaccharides (Asen et al., 1972). Besides the pigment and copigment molecular structure and their relative concentration, copigmentation may also be affected by the ionic

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strength, pH of the medium, solvent and temperature (Asen, Norris, & Stewart, 1972; Asen, Stewart, & Norris, 1971).

Although basic mechanisms related with copigmentation has been clarified to a considerable extent, many questions still remain, particularly related to the impact of polysaccharides on copigmentation mechanism. In fact, anthocyanins – polysaccharides interaction is a very important topic to be considered on anthocyanin's stabilization as these pigments are bio-synthesized in a polysaccharide rich environment. Anthocyanins are located primarily within the plant cell vacuoles in a free non-complexed form, enclosed by plant cell wall (CW) (Rodríguez et al., 2004). During fruit processing and beverages production, anthocyanins extraction requires CW rupture. When exiting from ruptured cells, anthocyanins and CW components come into contact with this interaction affecting anthocyanins extraction (Padayachee et al., 2012) and subsequent copigmentation associations, thus affecting the final color of food products, an important issue to be considered in the application of these pigments in the Food-Industry. The interaction between anthocyanins and polysaccharides is influenced by polysaccharide CW composition, composed essentially by hemicelluloses and pectin inter-knotted with the network of cellulose microfibrils. Cellulose and pectins in grape cell walls, accounts 30–40% of the polysaccharide components. Pectin is a group of polysaccharides rich in galacturonic acid (GalA) and classified in three types of polymers: the major homogalacturonan polymer (HG), and the two branched side chain polymers, containing rhamnosyl residue, the so-called rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Vicens et al., 2009).

Bearing this, the aim of this work was to assess the impact of low methoxylated pectic polysaccharide on the copigmentation interactions occurring between oenin (the main anthocyanin of red wine) and a common copigment such as catechin through the determination of the respective copigmentation constants (K_{CP}). The relationship between copigmentation ability and the formation of anthocyanin-pectin entities was also evaluated. Additionally, it was intended to monitor the interaction between oenin and catechin (in the absence and presence of pectin) using ^{1}H NMR experiments through the determination of dissociation constant (K_d).

2. Material and methods

2.1. Materials

Malvidin-3-*O*-β-D-glucoside (oenin, O) was extracted from a young red wine (*Vitis vinifera* L. c. v. Touriga Nacional) by semipreparative chromatography using a reversed-phase C18 column (250 mm × 4.6 mm i.d.), as described elsewhere (Pissarra, Mateus, Rivas-Gonzalo, Santos Buelga, & de Freitas, 2003). The purity of this pigment was assessed by HPLC-DAD and ^{1}H NMR (measured in D_2O on a Bruker Avance 600 spectrometer). (+)-Catechin (CP), low methoxylated pectic polysaccharide (LMP) from *citrus* fruits, citric acid, sodium citrate, sodium chloride, D_2O (99.9%), DCl (99%) and EtOD (99%) were purchased from Sigma-Aldrich (Madrid, Spain). NaOD (99.5%) was purchased from Euriso-top®. Pectin was purified by precipitation with ethanol and was analyzed by colorimetric methods and gas chromatography to determine sugar composition and esterification degrees, as described elsewhere (Coimbra, Waldron, & Selvendran, 1994; Nunes, Rocha, Saraiva, & Coimbra, 2006). According to these analyses it was found that pectin was composed by 85% galacturonic acid, 10% galactose and 5% of other sugars. The degree of methylation and acetylation was determined to be 14% and 1%, respectively. All aqueous solutions were prepared with distilled water.

2.2. Study design

This work involved experimental assays to allow the clarification of the effect of a cell wall polysaccharide (pectin) on anthocyanins copigmentation associations. Briefly, the copigmentation binding constants between oenin and catechin in the absence and presence of a cell wall polysaccharide were determined by UV–vis experiments (Sections 2.3) followed by the determination of the equilibrium constants for oenin and oenin-pectin complexes (Sections 2.4) through direct pH jumps. These determinations allowed to understand the possible impact of a pectic polysaccharide on anthocyanins color. This work also involved molecular techniques particularly NMR (Sections 2.5) and computational studies (Sections 2.6) to elucidate the impact of a pectic polysaccharide on oenin copigmentation association.

2.3. Copigmentation experiments

All samples were prepared in a citrate buffer solution (0.2 M) with 12% ethanol at pH 3.5 and the ionic strength was adjusted to 0.5 M by the addition of sodium chloride. Each pigment/copigment solution was prepared by mixing a volume of pigment solution (10^{-4} M) with an aliquot of copigment solution (10^{-2} M) to give the required pigment/copigment molar ratio (1:0, 1:5, 1:10, 1:20, 1:30 and 1:40). Aliquot of pectin solution (10 g/L) were added to give the required concentration (1.5, 2.5 and 5 g/L). The intermolecular copigmentation studies were performed at a low pigment concentration to minimize self-association effects (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009). Although ethanol largely reduces the copigmentation effect (Dangles & Brouillard, 1992), its inclusion in the experimental conditions was crucial to prevent precipitation during the spectroscopic analysis. Each experiment was performed in triplicate and all solutions were left to equilibrate for 30 min at room temperature before spectroscopic measurements. The absorbance values were collected at the maximum absorption wavelength of free oenin at pH 3.5 (λ_{max} 524 nm). Parameter *r*, which represents the ratio between the molar absorption coefficient of the complex (oenin/copigment molar ratio = 1:40) and the free flavylium ion (10^{-4} M), was determined in strongly acidic solutions (1 M aqueous HCl, pH ≈ 0) to ensure that the flavylium ion is the sole anthocyanin form.

2.3.1. UV/visible spectroscopy

After equilibration for 30 min, UV–visible spectra were recorded in 1 nm steps from 360 to 860 nm on a Bio-Tek Power Wave XS spectrophotometer, equipped with Gen5 Software. Spectroscopic measurements were performed in triplicate in a 1 cm path length cell and at a constant temperature (25 °C).

2.3.2. Data analysis

The curve fittings were carried out using the Scientist program (MicroMath, Salt Lake City, UT, USA). Curve fittings were achieved through a least-squares regression method. Statistical analysis reported standard deviations and correlation coefficients.

2.3.3. Binding free energies determination

The values of Gibbs free energy ($\Delta G°$) of the copigmentation reactions were determined by Eq. (1):

$$\Delta G^0 = -RT \times \ln K_{CP} \qquad (1)$$

where K_{CP} was the equilibrium constant for the reaction of the copigmentation, *R* was the gas constant (1.987 cal/mol K) and *T* the temperature (K).