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Examination of molecular mechanism for the enhanced thermal stability of anthocyanins by metal cations and polysaccharides

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ABSTRACT

Anthocyanins exhibit colour variation over wide pH range but the colour stability is relatively low at the physiological pH. To improve the stability of anthocyanins in neutral to weakly acidic pH region, effects of metal cations and polysaccharides on the colour stability of cyanidin-3-glucoside (C3G) were examined by ultraviolet–visible and resonance Raman spectroscopies. C3G was thermally stabilized by the addition of Fe^{3+} but formed aggregation. However, further addition of anionic polysaccharides enhanced the thermal stability of C3G without aggregation. Similar stabilisation was confirmed for delphinidin-3-glucoside (D3G) but not for pelargonidin-3-glucoside. The stability of anthocyanins considerably varied depending on pHs and kinds of metal cations, polysaccharides and buffer molecules. The characteristic resonance Raman bands of C3G–Fe^{3+} and D3G–Fe^{3+} complexes were significantly affected by the addition of alginate, $^{18}O/^{16}O$-isotope substitution, and Fe^{2+}/Fe^{3+}-replacement. These results suggest that alginate associates with C3G through Fe^{3+} to form a stable complex, which enhances the thermal stability of C3G.

1. Introduction

Anthocyanins are the most abundant flavonoid involved in plants and providing their colour variation (Jackman, Yada, Tung, & Speers, 1987; Welch, Wu, & Simon, 2008). Much attention has been paid to their anti-oxidative functions (Cao, Sofic, & Prior, 1997; Kahkonen & Heinonen, 2003; Rahman, Ichiyanagi, Komiyama, Hatano, & Konishi, 2006). Thus, anthocyanins are utilized for foods and beverages as natural colourants and anti-oxidants for beneficial effects of human health. The colour properties of anthocyanins are closely related with the molecular structures which are susceptible to pH changes of the solution, metal cations, and their substituents (Francis, 1989; Jackman et al., 1987). In the low-pH region (pH <3), anthocyanins exist as highly stable flavylium cations with bright red colour. As the pH increases, it changes to a colourless carbinol pseudo-base upon the hydrolysis, and undergoes ring opening to form a yellow chalcone. The thermal stability is largely deteriorated, particularly in the weakly acidic pH to neutral pH region.

Several factors enhancing the stability of anthocyanins are reported; acylation (Cevallos-Casals & Cisneros-Zevallos, 2004; Dyrby, Westergaard, & Stapelfeldt, 2001; Fossen, Cabrita, & Andersen, 1998; Kirca, Ozkan, & Cemeroglu, 2007), glucuronidation with enzyme, self-association, copigmentation (Asen, Stewart, & Norris, 1972; Bakowska, Kucharska, & Oszmianski, 2003; Bridle & Timberlake, 1997; Mazza & Brouillard, 1990; Osmani et al., 2009). Among the acylated compounds, polyacylated anthocyanins were reported to be more stable in neutral to weakly acidic pH region. It has been proposed that the higher stability of polyacylated anthocyanins is originated from sandwich-like intramolecular stacking due to hydrophobic interactions between aglycon and aromatic carboxylic acids. Such a stacking may prohibit hydration of the aglycon and degradation of the colour (Brouillard, 1983; Goto & Kondo, 1991). Although these polyacylated anthocyanins are highly stable, they are not commercially available and difficult to utilize as natural colourant for foods or beverages.

Anthocyanins associate with metal cations and flavons to form complicated supermolecules. Based on the crystal structure, commelinin was revealed to be a metalloanthocyanin composed of anthocyanin, flavone, and metal cation with a ratio of 6:6:2, respectively (Kondo et al., 1992). In addition, it was demonstrated that blue colours of cornflower pigment arise from a complex of six anthocyanins and flavones with four metal cations to form a supermolecular pigment (Shiono, Matsugaki, & Takeda, 2005). Several hydroxyl groups on the B-ring of anthocyanins were involved in the binding of metal cations. These metalloanthocyanins are

Abbreviations: C3G, cyanidin-3-glucoside; P3G, pelargonidin-3-glucoside; D3G, delphinidin-3-glucoside; MES, 2-morpholinoethanesulphonic acid; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; MOPS, 3-morpholinopropanesulfonic acid; Alg, alginate; Chi, chitosan; Pec, pectin; Car, ι-carrageenan; MC, methyl cellulose.

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self-assembled with a stoichiometric ratio, but readily dissociate in aqueous solution. However, it was reported that anthocyanin-metal chelates interact with some kinds of polysaccharides including pectin to enhance the colour stability of the complex (Bayer, 1966; Buchweitz, Carle, & Kammerer, 2012; Buchweitz, Nagel, Carle, & Kammerer, 2012; Lewis, Walker, & Lancaster, 1995). The information concerning the effects of polyacylation, metal-binding, and interactions with polysaccharides on the properties of anthocyanins indicate that metal cations are capable of binding anthocyanins and polysaccharides to form a stable complex, and such associations may protect the aglycons from hydration and stabilize them in the similar manner with polyacylated anthocyanins (Brouillard, 1983; Goto & Kondo, 1991).

In the present study, we examined effects of metal cations and ionic polysaccharides on the stability of anthocyanins under neutral to weakly acidic pH condition by ultraviolet–visible (UV–Vis) and resonance Raman spectroscopies. Cyanidin-3-glucoside was used as a model compound, and compared with the analogues, pelargonidin-3-glucoside and delphynidin-3-glucoside. In the presence of both Fe^{3+} and anionic polysaccharides, the thermal stability was maintained for cyanidin-3-glucoside and delphynidin-3-glucoside without aggregation but not for pelargonidin-3-glucoside. The stability of anthocyanins varied considerably depending on pHs and kinds of metal cations, polysaccharides, and buffer molecules. The interaction modes between cyanidin-3-glucoside and the cofactors were examined by resonance Raman spectroscopy. Based on these findings, the molecular mechanism of the enhanced thermal stability of cyanidin-3-glucoside by several cofactors is discussed.

2. Material and method

2.1. Materials and sample preparation

Cyanidin-3-glucoside (C3G, >96%), pelargonidin-3-glucoside (P3G, >97%), and delphynidin-3-glucoside (D3G, >97%) were purchased as chloride salts from Extrasynthese, and used without further purification. Sodium alginate (Alg), pectin (Pec, 3.0–7.0% of methoxyl group), and chitosan (Chi, 80.0 mol/mol% of deacetylation) were from Wako Pure Chemical Industries, and methyl cellulose (MC) and ι-carrageenan (Car) were from Tokyo Chemical Industry. $FeCl_3{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, $AlCl_3{\cdot}6H_2O$, and $CaCl_2{\cdot}2H_2O$ were commercially available (analytical grade, Wako Pure Chemical Industries). Good buffers (MES, Bis-Tris, and MOPS) and other buffers (glycine, acetate, citrate, propionate, succinate, lactate, tartrate, malate, and phosphate) were prepared using analytical grade reagents. To eliminate contamination, distilled and deionized water (Auto Still WG203, Yamato) was further purified with Simpli Lab (Millipore), and used as ultrapure water for all the sample preparations. C3G was dissolved in ultrapure water to be a concentration of 2.6 mM. This C3G stock solution was divided into 1.5 ml of cryogenic tube wrapped with aluminum foil and stored at −20 °C until use. Each sample tube was used for one set of experiments to prohibit sample degradation due to freeze–thawing. For each measurement, 30 μl of C3G, P3G, or D3G stock solution was diluted with the appropriate buffer solution, and mixed with 6 μl of 100 mM metal chloride stock solutions ($FeCl_3$, $FeCl_2$, $AlCl_3$, or $CaCl_2$) followed by the addition of 30 μl of 10 g/l polysaccharide stock solutions, which were prepared by dissolving the polysaccharide in ultrapure water with stirring at room temperature for 3–6 h and adjusted to pH 6.0 with 0.10 M HCl or NaOH solution. The final concentration of anthocyanin, metal cation, polysaccharide, and buffer molecule was 2.6×10^{-2} mM, 0.20 mM, 0.10 g/l, and 20 mM, respectively. For $^{18}O/^{16}O$-isotope substitution, 2 μl of C3G stock solution was diluted with 171 μl of $H_2{}^{18}O$ (>98%, Cambridge Isotope Laboratory), and then 20 μl of 200 mM MES, 2 μl of 20 mM $FeCl_3$, and 5 μl of 4.0 g/l alginate were added to become the final concentration of each component identical with that described above. The final content of $H_2{}^{18}O$ was calculated to be 85.5% (v/v). The sample solution was incubated at 0 °C for 12 h. Sample degradation during the incubation was not confirmed under the present condition.

2.2. Degradation studies

For thermal degradation, the fresh sample solution was maintained at 60 °C using a Dry Bath Incubator MD-01N (Major Science). The thermal stability of each sample solution was evaluated by monitoring the absorbance at each peak wavelength after the stirring of the sample solution incubated at 60 °C for 0, 2.5, 5.0, 10, 20, 40, and 80 min on a UV–Vis spectrophotometer UV-mini124 (Shimadzu). For thermal stability of the sample solutions at room temperature (25 °C), time profiles monitored at respective peak wavelengths were automatically recorded on a UV–Vis spectrophotometer U-3500 (Hitachi) with 30 s interval over 80 min.

2.3. Resonance Raman analysis

Resonance Raman spectra were recorded on a Holo Probe 532 (Kaiser Optical Systems) coupled to a Nd: YAG laser (JUNO 532-100L, Showa Optronics). The second harmonic generation (532 nm) was provided with the intensity of 10 mW at the sample surface, and the backscattering from each sample in a vial glass tube was collected at room temperature (25 °C) with a thermoelectrically cooled CCD detector through Kaiser super notch filters and optical fibers. The concentration of anthocyanins and cofactors in the sample solution was the same as that used for the degradation study. Each spectrum was obtained from a fresh sample to minimize the laser-induced degradation, and three spectra from different samples were averaged to improve the signal-to-noise ratio.

3. Results and discussion

3.1. Effects of Fe^{3+} and alginate on the thermal stability of C3G

Fig. 1A shows effects of addition of Fe^{3+} and alginate on the absorption property of C3G at room temperature and pH 6.0. The C3G spectrum (dotted line) exhibited typical absorption peaks at 531 nm and 429 nm. Upon the addition of Fe^{3+} (dashed line), these bands showed a bathochromic shift due to the interaction of C3G with Fe^{3+} (Buchweitz, Carle et al., 2012). Similar band shifts were observed by the addition of both Fe^{3+} and alginate (solid line) with further increase in the band intensity, but were not confirmed when only alginate was added (data not shown). Interestingly, the C3G spectrum in the presence of Fe^{2+} (dotted and dashed line) exhibited further red-shift and broadening, and was largely different from that in the presence of Fe^{3+} and alginate. This suggests that the redox change of Fe^{3+} upon the addition of alginate is unlikely. It is of note that contribution from alginate which has no specific band in this region (data not shown) and/or Fe^{3+} (thin solid line) to the characteristic band at around 570 nm is negligible. These results indicate that C3G interacts directly with Fe^{3+} and indirectly with alginate through Fe^{3+}. The effects of Fe^{3+} and alginate on the stability of C3G at room temperature were examined by monitoring time profiles of the relative peak intensity of C3G in the presence of Fe^{3+} or Fe^{3+} and alginate (Fig. 1B). The intensity was rapidly decreased in the absence of Fe^{3+} and alginate (dotted line). In contrast, the intensity was gradually increased and reached to a plateau level when both Fe^{3+} and alginate were involved (solid line). This increase in intensity implies that C3G has