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Isolation of strawberry anthocyanins using high-speed counter-current chromatography and the copigmentation with catechin or epicatechin by high pressure processing

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ABSTRACT

Three anthocyanins were isolated from strawberry extract by high-speed counter-current chromatography, using a biphasic mixture of *tert*-butyl methyl ether, *n*-butanol, acetonitrile, water and trifluoroacetic acid (2.5:2.0:2.5:5.0:1.0%). The anthocyanins were identified as pelargonidin-3-rutinoside, cyanidin-3-glucoside and pelargonidin-3-glucoside with purity of 95.6%, 96.2% and 99.3% respectively. Additionally, the copigmentation reaction rates between pelargonidin-3-glucoside and phenolic acids (catechin or epicatechin) at pH 1.5 and 3.6, pressure 0.1 and 500 MPa, and temperature 60 °C were calculated. The absorbance of pelargonidin-3-glucoside at pH 3.6, with high quantity of phenolic acids was significantly increased by high pressure. The complex of pelargonidin-3-glucoside/catechin has a binding energy of 78.64 kJ/mol at pH 3.6, and 39.13 kJ/mol at pH 1.5; pelargonidin-3-glucoside/epicatechin has a binding energy of 75.34 kJ/mol at pH 1.5 and 54.47 kJ/mol at pH 3.6. The hydrogen bond and van der Waals interaction were the main forces contributing to the structures of complex.

1. Introduction

Anthocyanin is a group of pigments in plant organs that are responsible for the orange, red, purple and blue of fruit, vegetable, and flower (Giusti & Wrolstad, 2003). There are more than 500 different anthocyanins in nature. However, only 6 anthocyanins are found in fruits and vegetables, including cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin (Kong, Chia, Goh, Chia, & Brouillard, 2003). As water-soluble colorants and antioxidants, anthocyanins have been successfully isolated by many techniques, including solid phase extraction, liquid-liquid extraction, counter-current chromatography, medium pressure liquid chromatography and high performance liquid chromatography (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The use of these natural colorants in the food industry is limited because of their relatively low stability to several processing and storage conditions. In fact, the stability of anthocyanin is easily influenced by pH, temperature, chemical structure, anthocyanin and copigment concentration, light, oxygen, enzymes, proteins and metallic ions (Rein, 2005).

Research involving the copigmentation reactions of anthocyanins revealed that inter- and intramolecular pigmentations, self-association, and metal complexation contribute to the stability of anthocyanins. The copigments could be anthocyanin, polyphenol, flavonoid, organic acid, nucleotide, and mental ion, which have planar structures or rich p-orbital electrons (Teixeira et al., 2013). As the colored forms of anthocyanins are almost planar structure, efficiently delocalized π-electrons and lacking of electrons, which could easily accept electronic transition from copigments (Castañeda-Ovando et al., 2009), and form a sandwich-like structure. The copigmentation force consists of hydrogen bond and van der Waals interaction (vertical π–π stacking) between the planar polarizable nuclei of anthocyanin. These non-covalent interactions could protect the anthocyanin molecule from water nucleophilic attack (García-Viguera & Bridle, 1999), lead to an increase in absorbance (hyperchromic effect) and a positive wavelength shift to the visible absorbance maximum (bathochromic shift) (Davis & Mazza, 1993).

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In addition, the copigmentation reactions are strongly affected by pH, temperature, copigment concentrations, solvents, and molecule structures (Teixeira et al., 2013). Higher copigmentation effect was observed with higher ration of copigment versus the anthocyanin, while higher temperature and the presence of methanol, formamide or salts could reduce the copigmentation effects. At very low pH (pH = 1), copigmentation reactions are much weaker than at pH 2–5 for the carbinol pseudobase. And the chalcone pseudobase cations could interact with copigments more easily than the flavylium cation (Rein, 2005). Moreover, the weak forces between anthocyanin and copigments are unstable and easily affected by high pressure processing (> 200 MPa) (Silva et al., 2014).

Anthocyanins, such as pelargonidin-3-glucoside and cyanidin-3-glucoside, contribute to the red color and antioxidant activity of strawberry (Tadapaneni et al., 2012). Although the concentration of anthocyanin as well as the color of strawberry juice were not significantly affected by high pressure processing (HPP) (Andrés, Villanueva, & Tenorio, 2016; Marszałek, Mitek, & Skąpska, 2015; Patras, Brunton, Da Pieve, & Butler, 2009), the presence of various anthocyanins, catechins, and predominant cations at specific pH could affect the copigmentation reactions, leading to color change under high pressure. Moreover, the effects of pressure and treatment time on the copigmentation interaction between anthocyanin and phenolic acids have not been discussed yet.

In order to explore the effects of HPP on the copigmentation of anthocyanin, high purity pelargonidin-3-rutinoside, cyanidin-3-glucoside, and pelargonidin-3-glucoside were isolated from strawberry extract using high speed counter current chromatography (HSCCC), and the copigmentation reaction rates between pelargonidin-3-glucoside (79.55% of total anthocyanin content) and phenolic acids (catechin or epicatechin) were investigated under high pressure processing. Additionally, the effects of high pressure and pH were also studied with theoretical calculations.

2. Materials and methods

2.1. Materials and chemicals

Strawberry fruit (Strawberry Tongzi I) was collected from Tianyi Biological Engineering Co., Ltd (Beijing, China), frozen immediately and stored at −20 °C. Catechin and epicatechin were purchased from Sigma-Aldrich (Shanghai, China). All the organic solvents used for crude extract preparation and HSCCC isolation were analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Acetonitrile and formic acid used for HPLC and ESI-MS/MS analysis were chromatographic grade and purchased from Sigma-Aldrich Co. LLC. Water with electrical resistivity of 18.2 MΩ was purified by an ultra pure water system (Elga, UK).

2.2. Preparation of crude extract

A sample of 500 g of strawberry was mixed with 2 L of methanol containing 1% trifluoroacetic acid and homogenized at 30,000 r/min for 10 min by high-speed homogenized (JHBE-50T, Jinding Co., Henan, China). Subsequently, the mixture was kept in the dark for 24 h at 4 °C. The supernatant was collected and filtered through a 0.22 μm filter (Millipore, USA) and concentrated by vacuum distillation (50 rpm, 500 Pa and 40 °C) until a final volume of approximately 0.1 L was reached. The extract was loaded to a macroporous resin column (23 cm × 2.6 cm, containing 100 mL Amberlite XAD-7 macroporous resin). After loading, the column was washed with 20 column volumes of water containing 0.5% trifluoroacetic acid (TFA) (1 L) at a flow rate of 2.0 mL/min, and eluted with 10 column volumes of methanol-water solution with a linear gradient of methanol percentage from 60 to 100%. The eluent was collected and evaporated with a rotary evaporator (50 rpm, 500 Pa and 40 °C). After evaporation, the dried anthocyanin extract was stored in the dark at 4 °C prior to HSCCC isolation and further analysis.

2.3. HPLC and MS analysis of anthocyanins of crude extract

For HPLC analysis, 50 mg of the crude extract was dissolved in 10 mL 0.5% TFA solution. The components in the extract were analyzed using the Shimadzu LC-20A liquid chromatography system (Shimadzu, Tokyo, Japan). The solvent system was composed of mobile phase A (water: formic 97:3, v/v) and mobile phase B (acetonitrile). Gradient conditions were as follows: 0–20 min, 10–20% B; 20–30 min 20% B. Column reequilibration time was 10 min. The flow rate was 1 mL/min, and 10 μL aliquots were injected onto a Venusil XBP C18 column (4.6 × 250 mm, 5 μm) (Agela Technologies, USA). Analytes were detected at 520 nm. Then all elutes were collected separately according to the absorbance and directly infused into the source at a flow rate of 1.0 μL/min via a syringe pump. The capillary was maintained at 550 °C and at a voltage of 3.0 kV. Mass scan (MS) and daughter spectra (MS/MS) were measured from m/z 200 to 700. Collision-induced fragmentation experiments were performed by collision gas at 0.5 mL/min, and the collision energy was set as 100. Mass spectrometry data were acquired in the positive mode by I CLASS XEVO TQ-S (Waters Corporation, USA).

2.4. HSCCC isolation procedure

A 40 mL initial solvent system consisting of different ratios of *tert*-butyl methyl ether, *n*-butanol, acetonitrile, water and TFA was prepared in a centrifuge tube. Then, the tube was sealed and shaken to equilibrate the solvent system. The solvents were continuously distributed until two phases formed. About 10 mg of crude dried extract was added to the solvent system and thoroughly shaken to reach equilibrium. Identical volumes of the upper and lower phases were transferred to HPLC vials and analyzed. The k value (the amount of solute in the stationary phase divided by that of the mobile phase) and separation factor α (the ratio of every two k values) were calculated. The solvent systems were adjusted to meet the criteria of the k ($0.5 \leq k \leq 2.0$) by changing the volume ratios (Ito, 2005). After the solvent system was selected, about 2.4 L optimized solvent system, composed of *tert*-butyl methyl ether, *n*-butanol, acetonitrile, water and trifluoroacetic acid, was prepared in a separatory funnel and equilibrated by shaking at room temperature. Then the upper and lower phases were degassed for 20 min and allowed to stand for 4 h. Meanwhile, the sample solution was prepared by dissolving 70 mg of dried crude extract in 10 mL of the two-phase solvent system. The crude extract was separated using a TBE-300C (Shanghai Tauto Biotechnology Corporation, Shanghai, China) and the HSCCC preparation was performed as follows: the coiled column was filled with the upper phases (stationary phase) of the solvent system at 10.0 mL/min and the lower phase (mobile phase) was pumped into the column at a flow rate of 2.0 mL/min, then the apparatus was rotated at 850 rpm. After a hydrodynamic equilibrium was reached, a 10 mL sample aliquot was injected into the sample loop. The eluent was monitored with a UV detector at 520 nm, and the peak fractions were collected according to the chromatographic profile using data recording software (Shanghai Tauto Biotechnology Corporation).

2.5. Analysis of compounds from HSCCC procedure

The UV–vis spectra were recorded by a UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan) at a temperature of 25 ± 3 °C from 400 to 700 nm using a 1 cm path length cell. The collected compounds were analyzed by HPLC, and the purity was calculated by the target analyte peak area divided by the total peak area (unitary area method). The structural identification of compounds was performed by solari X Qq-FTMS mass spectrometer (SolariX hybrid Qq-FTMS, Bruker, Daltonics, Germany). All compounds were directly infused with an acetonitrile: