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Effects of high hydrostatic pressure-assisted organic acids on the copigmentation of *Vitis amurensis Rupr* anthocyanins

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Chemical compounds studied in this article::
Ferulic acid (PubChem CID: 445858)
D-Gluconic acid (PubChem CID: 10690)
Caffeic acid (PubChem CID: 689043)
Vanillic acid (PubChem CID: 8468)
Delphinidin-3-*O*-glucoside (PubChem CID: 165558)
Cyanidin-3-*O*-glucoside (PubChem CID: 197081)
Petunidin-3-*O*-glucoside (PubChem CID: 176449)
Peonidin-3-*O*-glucoside (PubChem CID: 14311152)
Malvidin-3-*O*-glucoside (PubChem CID: 11249520)

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ABSTRACT

Natural anthocyanins are safer and nutritious as compared to synthetic pigments; however, their stability is poor. They can produce spontaneous copigmentation with organic acids, leading to the improvement of colour stability, albeit slowly. Box–Behnken experimental design was used to elucidate the mechanism of copigmentation between *Vitis amurensis Rupr* anthocyanins (0.1 mg/mL) and organic acids (0.87 mg/mL, ferulic acid:D-gluconic acid:caffeic acid:vanillic acid = 1.5:2.5:2.5:0.5, w/w/w/w) promoted by high hydrostatic pressure (HHP; 300 MPa, 2 min). The copigmentation effect and antioxidant activity of anthocyanins were also evaluated. The structure of anthocyanins was analysed using ultraviolet–visible spectroscopy, Fourier-transform infrared spectroscopy, high-performance liquid chromatography, and mass spectrometry. The results of HHP copigmentation showed that the following anthocyanins were newly formed—delphinidin-3-*O*-catechol, petunidin-3-*O*-catechol, delphinidin-4-vinyl-catechol, petunidin-3-*O*-guaiacol, malvidin-4-vinyl-guaiacol, cyanidin-3-*O*-(6″-*O*-caffeoyl)-glucoside, peonidin-3-*O*-(6″-*O*-caffeoyl)-glucoside, delphinidin-3-*O*-(6″-*O*-caffeoyl)-glucoside, malvidin-3-*O*-glucoside-4-vinyl-guaiacol, and malvidin-3-*O*-(6″-*O*-feruloyl)-glucoside—owing to appropriate modifications that increased the copigmentation rate (R = 42.12%), photo-thermal stability (R > 45%), and potential antioxidant activities expressed *in vivo* ($p < 0.01$ *vs.* Model Group).

1. Introduction

Anthocyanins are water-soluble natural pigments categorised as flavonoids. They are glycosylated derivatives of 3,5,7,4′-tetrahydroxy flavylium cation, and different substituents exist on the B ring to form different classes of anthocyanins. Six classes that occur frequently include pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Fig. S1). The hydroxyl groups on positions 3, 5, and 7 of anthocyanins can form various anthocyanins by glycosidic linkage with one or more monosaccharides, disaccharides, or trisaccharides. The hydroxyl groups of the sugar molecules in the anthocyanins can also be combined with one or more organic acids such as *p*-coumaric acid, ferulic acid, and caffeic acid to form acylated anthocyanins (Aceve De la Cruz et al., 2012; Chiou, Panagopoulou, Gatzali, Marchi, & Karathanos, 2014; Veberic, Slatnar, Bizjak, Stampar, & Mikulic-Petkovsek, 2015).

Anthocyanins are widely found in natural plants, especially in grapes and blueberries (Chen, Zhao, Tao, Zhang, & Sun, 2015; Fraige, Pereira-Filho, & Carrilho, 2014). Among all grape varieties, *Vitis amurensis Rupr* has the darkest skin colour, the richest natural anthocyanin content, and the best cold resistance. It has been planted in Ji'an and Liuhe in northeastern China for more than 40 years and cultivated in more than 1400 ha, with an annual output of more than 20,000 tons, exceeding the cultivation area of blueberries (Liang et al., 2014; Song et al., 2002). *V. amurensis Rupr* anthocyanins not only render an attractive colour to the products, but also have health attributes such as softened blood vessels, improved antioxidant activity, skin luster, and

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reduced cancer risk (Fratantonio et al., 2017; Lee et al., 2016). They have a remarkable potential for application in food processing and can be used as a colorant instead of synthetic red pigments in the production of beverages, fruit wine, and other products, because it is safe and nutritious. However, natural anthocyanins are unstable as they are easily affected by temperature, light, and pH. They are often degraded during extraction, processing, and storage, which remarkably limits their application (Lago-Vanzela et al., 2014; Reque, et al., 2014; Sui, Bary & Zhou, 2016). Therefore, many studies have been focusing on improving the stability of natural anthocyanins.

The copigmentation effect is an effective way to improve the colour intensity and stability of natural anthocyanins by interacting with copigments (Cortez, Luna-Vital , Margulis, & Elvira, 2016; Guan & Zhong, 2015; He, et al., 2016). At present, the conventional copigmentation technology involves the effectuation of anthocyanins and copigments via covalent bonds, hydrogen bonds, Van der Waals forces, and other forms to produce copigmentation spontaneously. The naturally extracted organic acids such as caffeic acid and sinapic acid are used as copigments owing to their high safety and good copigmentation effect; however, spontaneous copigmentation requires a prolonged duration and is less efficient (Gris, Ferreira, Falcão, & Bordignon-Luiz, 2007a, 2007b; Marković, Petranović, & Baranac, 2005; Zhang, He, Zhou, Liu, & Duan, 2015) and cannot fulfil production needs. High hydrostatic pressure (HHP) is a non-thermal processing technology that can affect the spatial arrangement of molecules and catalytic reaction in a short period. It has many advantages such as instantaneous compression, uniform action, safe operation, and eco-friendliness. Thus, HHP has a wide range of applications such as extraction of effective substances and sterilisation (Huang, Hsu, Yang, & Wang, 2013; Marszałek, Woźniak, Skąpska, & Mitek, 2017). It can be used to accelerate potentially the esterification and polymerisation reactions in wine as well as the aging process while maintaining the colour and quality of wine (Santos et al., 2016; Tao et al. 2016). Thus, HHP can be used to promote rapid copigmentation reaction between anthocyanins and organic acids, and thus, enhance the stability of anthocyanins.

As described previously, *V. amurensis Rupr* contains abundant anthocyanins that have numerous health benefits. However, natural anthocyanins are unstable, and the conventional copigmentation method is time-consuming and inefficient. Thus, this study aimed to evaluate the copigmentation effect of HHP-assisted organic acids on the *V. amurensis Rupr* anthocyanins and to explore the underlying copigmentation mechanism.

2. Materials and methods

2.1. Reagents and chemicals

Ferulic acid, D-gluconic acid, caffeic acid, and vanillic acid were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Concentrated hydrochloric acid (37%, w/v) and ethanol were purchased from Beijing Chemical (Beijing, China). Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin, cyanidin, petunidin, peonidin, and malvidin were obtained from ChromaDex (Irvine, USA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid were procured from Jinsui Biotechnology Co., Ltd. (Shanghai, China). Methanol and formic acid were obtained from Thermo Fisher (Waltham, USA). Superoxide dismutase (SOD) assay kit, glutathione peroxidase (GSH-PX) assay kit, malondialdehyde (MDA) assay kit, and Coomassie blue protein assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Materials and pre-treatment

V. amurensis Rupr ‘Beibinghong’ was collected from Liuhe County (Tonghua Wine Institute, Tonghua, China) on 28 September 2015. Liuhe County is located in the east longitude 125°17′–126°35′ and latitude 41°54′–42°35′, with an annual average temperature of 4.4–5.5 °C, annual minimum temperature of -36.2 °C, frost-free period of 126–140 d, activity accumulated temperature of 2800.2 °C, annual precipitation of 700–900 mm, and sunshine for 2550 h. The test garden soil is sandy with a pH 5.5–6.7. The planting area of Beibinghong is up to 400 ha with a yield of 15–23 t/ha. About 5.000 ± 0.005 kg *V. amurensis Rupr* was removed from the stalk and beaten using a comminuter, followed by grinding in a colloid mill for 15 min to make the particle size ≤ 50 μm for standby application. As the liquid [37% HCl-ethanol-distilled water (v/v/v = 1:65:34)]-to-solid ratio was 8.5:1 (w/w), and LDT-200/10–20.1 High-voltage Pulsed Electric Field Extraction Device (Huadi Biotechnology Development Co., Ltd., Changchun, China) was used to extract the anthocyanins at 15 kV/cm field strength for 4 pulses. After the supernatant was centrifuged at 1720 × *g* for 10 min, it was vacuum concentrated at 40–50 °C until the concentration density was (1.0–1.1) × 10^3 kg/m^3. Subsequently, the product was purified using D101 macroporous resin (4 °C), eluted using ethanol, concentrated under reduced pressure (under the same conditions as those for vacuum concentration), and lyophilized to obtain the lyophilized powder of anthocyanins (anthocyanin content, ≥25%, moisture content, ≤10.0%) from *V. amurensis Rupr* (He, Wen, Liu & et al., 2017; He, Wen, Yu & et al., 2017).

2.3. HHP copigmentation study

2.3.1. Selection of organic acids

Organic acids (ferulic acid, caffeic acid, vanillic acid, coumaric acid, D-gluconic acid, gallic acid, protocatechuic acid, fumaric acid, lactic acid, tannic acid, malic acid, succinic acid, and cinnamic acid) and lyophilized powder of anthocyanins were solubilized in ethanol and diluted with buffer (0.2 mol/L disodium hydrogen phosphate in 0.1 mol/L citric acid buffer solution, pH 3.0). The mixtures of organic acids (0.25 mg/mL) and anthocyanins (0.1 mg/mL) were reacted at 300 MPa pressure for 2 min by using a 600 MPa/30 L High Hydrostatic Pressure Food Processing Device (Kefa High-Pressure Technology Co., Ltd., Baotou, China). The anthocyanins without HHP and organic acid treatment (CK, untreated anthocyanins) and anthocyanins treated with HHP without organic acids (CK_1) were used as control. The copigmentation effect of different organic acids on anthocyanins under photo-thermal conditions was investigated in a shaking water bath at 100 °C for 1 h and under natural light at room temperature (25 ± 1 °C) for 10 days. The reaction mixture was centrifuged at 1720 × *g* for 10 min, and the absorbance of the initial copigmentation, heating treatment, and natural light treatment was determined using a T6 UV/vis spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing, China) at 521 nm. The absorbance of the three groups was compared to select organic acids with superior stability.

2.3.2. Optimum design of organic acid concentration

Four organic acids (ferulic acid, D-gluconic acid, caffeic acid, and vanillic acid) and three concentrations (0.05, 0.15, 0.25, and 0.1 mg/mL anthocyanins) were optimized using an $L_9(4^3)$ orthogonal design. The optimum concentration ratio of the compound organic acids was determined at 521 nm.

2.3.3. Response surface optimization design of HHP copigmentation

According to the results of the pre-experiment, the organic acid concentration A (0.35, 0.70, and 1.05 mg/mL compound organic acids, 0.1 mg/mL anthocyanins), copigmentation pressure B (250, 300, and 350 MPa), and pressurization time C (1, 2, and 3 min) were selected using Box–Behnken design. The copigmentation effect of the HHP was evaluated by measuring the absorbance at 521 nm.