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Interaction between *Vaccinium bracteatum* Thunb. leaf pigment and rice proteins

Li Wang [a,1], Yuan Xu [a,1], Sumei Zhou [b,1], Haifeng Qian [a,*], Hui Zhang [a], Xiguang Qi [a], Meihua Fan [c]

[a] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[c] Jiangsu Institute of Poultry Sciences, Yangzhou 225125, China

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ABSTRACT

In this study, we investigated the interaction of *Vaccinium bracteatum* Thunb. leaf (VBTL) pigment and rice proteins. In the presence of rice protein, VBTL pigment antioxidant activity and free polyphenol content decreased by 67.19% and 68.11%, respectively, and L^* of the protein–pigment complex decreased significantly over time. L^* values of albumin, globulin and glutelin during 60-min pigment exposure decreased by 55.00, 57.14, and 54.30%, respectively, indicating that these proteins had bound to the pigment. A significant difference in protein surface hydrophobicity was observed between rice proteins and pigment–protein complexes, indicating that hydrophobic interaction is a major binding mechanism between VBTL pigment and rice proteins. A significant difference in secondary structures between proteins and protein–pigment complexes was also uncovered, indicating that hydrogen bonding may be another mode of interaction between VBTL pigment and rice proteins. Our results indicate that VBTL pigment can stain rice proteins with hydrophobic and hydrogen interactions.

1. Introduction

The genus *Vaccinium* is widely distributed. Its members are found primarily in the Northern hemisphere, especially in mountainous tropical Asia and Central and South America (Fang & Peter, 2015; Xie, 2005). Many *Vaccinium* species, such as blueberry, cranberry and lingonberry, have been extensively investigated (McKay, Chen, Zampariello, & Blumberg, 2015; Rodriguez-Mateos, Cifuentes-Gomez, George, & Spencer, 2013; Viljanen, Heiniö, Juvonen, Kössö, & Puupponen-Pimiä, 2014). A number of anthocyanins, polyphenols and phenolic acids have been identified in these plants. These compounds are generally recognized for their potential biological activities, which include induction of apoptosis, inhibition of breast cancer and metastasis, postponement of aging, and regulation of blood glucose and lipids (Mahbub et al., 2013; Prior et al., 2010; Rendeiro et al., 2012). Pigments in *Vaccinium* plants have also been thoroughly investigated (Lee, Finn, & Wrolstad, 2004; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002), thereby revealing some of their properties and mechanisms of biosynthesis and staining (Kamiya, Yanase, & Nakatsuka, 2014).

Vaccinium bracteatum Thunb., known as Wu Fan Shu or Nan Zhu in traditional Chinese medicine, is distributed throughout China. Extensive information about *V. bracteatum* has recently been published in China, including its components (Chen & Zhang, 2014), functions (Wang, Zhang, Zhang, Yao, & Zhang, 2010) and utilization (Han et al., 2013). Although the species is still underused, *V. bracteatum* leaf (VBTL) pigment is used to dye rice in parts of eastern coastal China (Xu, Wang, Li, & Chen, 2013). While pigments of *Vaccinium* plants have been extensively studied, only preliminary research has been conducted on VBTL pigment, including its extraction (Wang, Jiang, Zhang, & Yao, 2008), purification (Langhansova, Landa, Marsik, & Vanek, 2012; Wang & Yao, 2006), physical and chemical properties (Wang, Xu, & Yao, 2011) and biological activities (Wang et al., 2010, 2013). Researchers have used VBTL pigment to dye white hair, fresh eggs and rice (Hu, Jiang, & Zhang, 2001; Jiang & Chen, 1999). Polyphenols have been found to be the main components responsible for the staining activity (Wei, Liu, Xu, He, & Zhang, 2007; Yu, Chen, & Pang, 2007), but the underlying mechanism is still unclear and the pigment is consequently underutilized.

Although some studies have addressed the interaction between polyphenols and proteins (Hudson, Ecroyd, Dehle, Musgrave, & Carver, 2009; Kanakis et al., 2011), the underlying binding

* Corresponding author at: State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
E-mail address: qianhaifeng@jiangnan.edu.cn (H. Qian).
[1] These authors contributed equally to this study.

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mechanisms have not been elucidated. This information is accordingly also lacking for the polyphenols of VBTL pigment and rice proteins. In light of the absence of relevant studies, we aimed to investigate the interaction between VBTL pigment and rice proteins using a combination of proteomic and biochemical strategies. We developed a simplified model system in which pigment was incubated with isolated classified rice proteins. We measured changes in VBTL pigment polyphenol content and antioxidant activity during the interaction and applied proteomic strategies, such as surface hydrophobicity and Fourier-transform infrared spectroscopy (FT-IR), to monitor various aspects of the rice proteins. The data acquired in this manner allowed us to characterize interaction patterns between VBTL pigment and rice proteins in detail.

2. Materials and methods

2.1. Reagents and standards

Leaves of *V. bracteatum* were supplied by the Jiangsu Jiujiu Environmental Science and Technology Co. (Wuxi, Jiangsu, China). Rice protein was purchased from King-N Rice Industry Group (Yichun, Jiangxi, China). All other chemicals used in this study were of analytical grade.

2.2. Isolation of rice proteins

Protein fractions were extracted according to the Osborne method. Rice protein samples were extracted twice with distilled water for 60 min at 25 °C. After centrifugation of each extract at 3000*g* for 30 min, the supernatant was used for the determination of the water-soluble protein, albumin. The residue was then extracted successively in a similar manner with 5% NaCl, 70% ethanol and 0.05 M NaOH. The supernatants of each extract were collected separately and used to estimate the salt- (globulin), alcohol- (prolamin) and alkali- (glutelin) soluble fractions. Albumin, globulin and glutelin were concentrated by isoelectric sedimentation at pH 4.1, 4.3 and 4.8, respectively, while prolamin was concentrated by evaporation. Residue remaining after the successive extractions corresponded to the insoluble proteins. Each fraction was collected and lyophilized by a lyophilizer (ACPHA1-4, CHRIST, Germany). The protein content of each fraction was determined by the Kjeldahl method (total nitrogen × 5.95).

2.3. Preparation of VBTL pigment

VBTLs were dried in a forced-air convention oven at 35 °C for about 24 h until a moisture content below 10% was reached. The leaves were then ground to a 40-mesh power and stored at 4 °C in a refrigerator until analysis.

The VBTL powder (100 g) was transferred to a dark-colored flask and mixed with 1000 ml of 40% (v/v) ethanol in a constant-temperature bath at 40 °C for 2 h. The ethanolic extract was then centrifuged at 4 °C and 3000*g* for 20 min. Afterwards, the supernatant was collected and the residue was re-extracted twice. Combined supernatants were evaporated to dryness *in vacuo* at 40 °C using a vacuum concentrator and then lyophilized (Alpha 1-4, Christ, Osterode, Germany). The crude VBTL pigment was stored at 4 °C until further purification.

Crude VBTL pigments (5 g) was dissolved in 50 ml water and loaded onto a chromatographic column (35 × 400 mm) packed with AB-8 macroporous resins. The column was washed with distilled water and 20% (v/v) ethanol until the eluent was colorless. The column was then eluted with 60% (v/v) ethanol at a flow rate of 1 ml min^{-1}. The eluent was collected and concentrated to completely remove the ethanol and then loaded onto another chromatographic column packed with AB-8 macroporous resins. This column was eluted with ethyl acetate at a flow rate of 1 ml min^{-1}. The eluent was collected and concentrated until the ethyl acetate was completely removed and then lyophilized. The VBTL pigment was finally stored at 4 °C for use in staining experiments.

Purified VBTL pigments (0.1 g) was dissolved in 50 ml distilled water prior to the addition of 2 g of each rice protein fraction. Pigment and proteins were allowed to interact at 70 °C for 60 min using a lab mixer. The resulting complex was filtered using No. 40 Whatman filter paper and washed with excess distilled water to remove redundant pigment before lyophilization. The freeze-dried complex was stored at 4 °C for further analysis. Rice protein (2 g) was dissolved in 50 ml distilled water and then incubated at 70 °C for 60 min. The resulting protein was treated by the same way as the pigment–protein complex. Measured polyphenol, protein and carbohydrate contents of the lyophilized powder were 61.97, 1.12 and 35.64%, respectively.

2.4. Color measurements

Color of powdered proteins and pigment–protein complex was measured with a high-precision color-measuring spectrometer (HunterLab, Reston, Virginia, USA), with the data generally represented by CIE Lab color space system parameters. In this system, each point in three-dimensional Cartesian space represents a different color, with its L^*, a^* and b^* values respectively corresponding to its position along black-to-white, green-to-red and blue-to-yellow axes. The distance between two measured points is termed the color aberration, represented as ΔL^*, Δa^* and Δb^*.

2.5. Determination of antioxidant activity and free polyphenol content

VBTL antioxidant activity was determined by a 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid assay according to the method of Pellegrini, Del, Colombi, Bianchi, and Brighenti (2003). Results were expressed in terms of Trolox equivalent antioxidant capacity (mmol of Trolox per kg [solid foods and oils] or L [beverages] of sample). Polyphenol content was determined by the Folin–Ciocalteu colorimetric method (Cai et al., 2010), with the results expressed as gallic acid equivalents in mg per g of extract.

2.6. Measurement of protein surface hydrophobicity

Protein surface hydrophobicity (S_0) was determined using 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe. The proteins were dispersed into phosphate buffer (pH 7.0, 10 mmol/L) and the isolates were serially diluted with the same buffer in concentrations of 0.0005% to 0.015% (w/v). Ten microliters of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 2 ml of diluted protein solutions, and the final concentration of ANS was 0.04 mM. Fluorescence intensities (FIs-the height of the emission peak at the wavelength of emission maximum) of ANS–protein conjugates were measured with an Aminco-Bowman spectrofluorometer (Hitachi, Tokyo, Japan). Excitation and emission wavelengths were 390 nm and 470 nm, respectively. The slopes of the plots of net FI versus percent protein were calculated by least squares linear regression and specified as S_0.

2.7. Analysis of secondary structure

The secondary structures of pigment–albumin, pigment–globulin, pigment–glutelin, and each protein were analyzed at different staining times (0, 4, 8, 12, 20, 40 and 60 min) by FT-IR following the method of Nabet and Pezolet (1997). Briefly, the solid powder was measured by IS10 fourier transform infrared spectrometer