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Digestion property and synergistic effect on biological activity of purple rice (*Oryza sativa* L.) anthocyanins subjected to a simulated gastrointestinal digestion *in vitro*

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ABSTRACT

Anthocyanins, a group of polyphenolic pigments, have been proved to possess various bioactivities. However, they are unstable in the small intestine and absorbed with low bioavailability. The discrepancy between the low bioavailability of anthocyanins and their good bioactivities has not been illuminated yet. Moreover, information about the digested property and stability of purple rice anthocyanins in the alimentary tract is still limited. Thus, the present work was designed to study the digestion property and the changes in antioxidant and cytoprotective activities of purple rice anthocyanins using an *in vitro* digestion model, and to investigate the interactions between gastric and intestinal digested anthocyanins. The results showed that anthocyanins amount and antioxidant and cytoprotective effects didn't change significantly during gastric digestion. However, about 76% of total anthocyanins were degraded during intestinal digestion. The IC_{50} values of intestinal digested sample tested by DPPH and ABTS methods were about 19.1 and 16.9 μg/mL, respectively, far higher than that of non-digested sample (about 7.7 and 7.1 μg/mL, respectively). Cytoprotective effect of intestinal digested sample also decreased significantly. Synergistic effects on antioxidant and cytoprotective activities were observed between the gastric and intestinal digested samples at a relative low concentration. Those results suggest that the bioactivities of purple rice anthocyanins may be changed after digestion and enhanced through the synergies between their gastric and intestinal digested catabolites.

1. Introduction

Previous studies have suggested that diets rich in fruits, cereals and vegetables can reduce the risk of some chronic diseases, such as diabetes and cardiovascular disease (Dani et al., 2008; Guo et al., 2008; Tanaka et al., 2010; van Dam, Naidoo, & Landberg, 2013). Nowadays, reducing the risks of diseases by plant-derived foods has gradually attracted widespread attention due to their low toxicity and few side effects. Substantial efforts have been made by food scientists and nutritionists to find bioactive substances from edible plants with potential health-promoting activity.

Anthocyanins, a group of water-soluble plant pigments with a flavonoid structure, are widely distributed in many plants, such as fruits, cereals and flowers (Hou, Qin, & Ren, 2010; Prior et al., 2008). Numerous *in vitro* and *in vivo* studies have proved that anthocyanins possess various bioactivities, *e.g.* anti-atherosclerosis, prevention of diabetes, and amelioration of light-induced retinal damage (Abdel-Moemin, 2011; Dani et al., 2008; Guo et al., 2008; Tanaka et al., 2010). The benefits of anthocyanins for human health seem to be unquestionable; and their potent antioxidant activity is considered to play a crucial role (Hou et al., 2013). However, a number of studies reported that intestinal absorption of anthocyanins is just around 1% of the orally ingested amount (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Although, gastric absorption of anthocyanins has also been found and about 20% anthocyanins can be absorbed through this way (Felgines et al., 2006; Fernandes, Faria, Calhau, De Freitas, & Mateus, 2014). The total absorption of anthocyanins may still be too low to perform their bioactivity *in vivo*, according to the results obtained from *in vitro* studies. The effective concentrations of anthocyanins used in the *in vitro* studies were always far higher than plasma anthocyanin concentrations (Hou et al., 2013; Tanaka et al., 2010; Wang, Zhang, Liu, Wang, Liu, & Ji, 2014). The discrepancy between the low bioavailability of anthocyanins and their good bioactivities has always made the scientific community feel confused. Therefore, the study of this paradoxical question is very necessary and meaningful. According to the chemical features of anthocyanins, they are unstable in the weak alkaline condition of the small intestine and degraded to some other metabolites, which is partly responsible for their low bioavailability (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002), since some

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bioactive compounds may synergize with each other when mixed together and can perform their bioactivities even at a low concentration (Amoros, Simõs, Girre, Sauvager, & Cormier, 1992; Cai et al., 2012). So, we hypothesized that the potential synergy may occur between anthocyanins and their digested metabolites to enhance their bioactivities, which may be an answer to the anthocyanins paradox.

Purple rice (*Oryza sativa* L.), mainly cultivated in Southeast Asia, is a colored variety of rice. Recently, a number of studies have shown that the bran of purple rice is highly enriched with a purple-black pigment (anthocyanins) and has a lot of bioactivities, including antioxidant (Chiang et al., 2006; Jang & Xu, 2009), hepatoprotective (Hou et al., 2013; Hou et al., 2010), anti-diabetic (Guo et al., 2008), and anti-inflammatory effects (Hu, Zawistowski, Ling, & Kitts, 2003). Although the chemical composition and biological activities of purple rice have been studied, information about the digestion property and stability of its anthocyanins in the alimentary tract is still limited. Therefore, one of the purposes of the present study is to investigate the digestion property and stability of purple rice anthocyanins using a simulated gastrointestinal digestion model *in vitro*. Another aim is to evaluate the changes in antioxidant and cytoprotective activities of digested purple rice anthocyanins and to determine whether there was a potential synergy between gastric digested and intestinal digested samples.

2. Materials and methods

2.1. Chemicals and reagents

2-Diphenyl-1-picryhydrazyl (DPPH) radical, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), methylthizol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), pepsin from porcine gastric mucosa (3860 units/mg protein) (EC 3.4.23.1), pancreatin from porcine pancreas (8× USP specifications), and bile salts were purchased from Sigma-Aldrich (Shanghai, China). Authentic standards of peonidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, and cyanidin-3-rutinoside were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Fetal bovine serum (FBS), penicillin, streptomycin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY). Annexin V-FITC/PI apoptosis kit was purchased from Beijing 4A Biotech Co., Ltd. (Beijing, China).

2.2. Extraction of purple rice anthocyanins

Purple rice was purchased from a local market in Mojiang county, Yunnan province, China. The method for preparing purple rice anthocyanins was according to previous report (Zou, Wang, Gan, & Ling, 2011) with minor modification. In brief, purple rice was ground using the HK-06A high-speed grinder (Changsha, Hunan, China) and then passed through an 80 mesh sieve. Thereafter, the powder was ultrasonically extracted three times with 80% acidified methanol containing 1% (v/v) formic acid at 40 °C for total 1 h (20 min for each extraction) with the ratio of material to liquid at 1:10 (w/v). Slurry of each extraction was filtered using a moderate-speed type of filter paper after being cool to room temperature. Then combined filtrates were evaporated under reduced pressure at 40 °C and lyophilized to get crude purple rice anthocyanins.

2.3. In vitro simulated digestion process and purification

In vitro simulated digestion was performed according to the protocol outlined previously (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Correa-Betanzo et al., 2014) with some modification. Briefly, 5 g crude purple rice anthocyanins were dissolved in acidified distilled water (final pH = 2, 1 M of HCl). Thereafter, porcine pepsin from porcine gastric mucosa (12,000 Units, EC 3.4.23.1, purchased from Sigma-Aldrich, Shanghai, China) was added into the sample and mixture was incubated in a shaker (37 °C, 200 rpm) under a dark condition for 2 h. After that, a portion of mixture was collected and stored at −80 °C as crude gastric digested sample. The remaining portion was used for intestinal digestion, which was firstly adjusted to pH 7.5 with 1 M of $NaHCO_3$. Then pancreatin (8× USP specifications) from porcine pancreas (1 mL, 2 g/L) and bile salts (1 mL, 25 g/L) were added, and mixture was incubated at the same condition as gastric digestion for other 2 h. At the end of the intestinal digestion, the pH of mixture was adjusted to pH 2 and also stored at −80 °C as crude intestinal digested sample.

In order to facilitate subsequent analysis and comparative experiments, anthocyanins in crude extract and each digested mixture were purified by AB-8 resin (Hou et al., 2013) to remove sugar, salts, or digestive enzymes. Briefly, sample was loaded onto AB-8 resin for 2 h. The AB-8 resin was firstly washed with 1% (v/v) formic acid aqueous solution for 5 column volume, and then eluted with 80% acidified methanol to obtain absorbed anthocyanins. The methanol eluent was evaporated and lyophilized to get anthocyanins powder. Those samples were stored at −20 °C and used in the subsequent analysis and experiments.

2.4. Spectral scan and total anthocyanin content estimation

Samples from non-digestion and *in vitro* digestion were scanned using Shimadzu UV–visible spectroscopy (Shimadzu UV-2550, Kyoto, Japan) to determine the stability of purple rice anthocyanins based on the absorbance at wavelengths ranging from 200 to 800 nm at pH 2 (Correa-Betanzo et al., 2014; Liu et al., 2014).

The spectrophotometric pH differential method (Hosseinian, Li, & Beta, 2008; Lee, Durst, & Wrolstad, 2005) was applied to estimate total anthocyanin content. Sample was firstly diluted 10 times with 0.03 M potassium chloride buffer (pH 1) and 0.4 M sodium acetate (pH 4.5), respectively. Then, the absorbance of each dilution was measured at 520 and 700 nm against distilled water as blank, respectively, using Shimadzu UV–visible spectroscopy (Shimadzu UV-2550, Kyoto, Japan). The total anthocyanin content of each sample was calculated as cyanidin 3-glucoside equivalents (mg/g) = $(A \times MW \times DF \times V \times 10^3) / (\varepsilon \times L \times m)$, where $A = (A_{520}–A_{700})_{pH\,=\,1}–(A_{520}–A_{700})_{pH\,=\,4.5}$, MW = 449.2 g/mol for cyanidin 3-glucoside, and ε is the extinction coefficient of cyanidin 3-glucoside = 26,900 L/mol/cm. DF is the dilution factor, in the present study, DF = 10, V is the volume of sample stock solution (L), L = 1 cm, and m is the tested sample weight (g). All samples were determined in triplicate.

2.5. HPLC analysis of anthocyanins

An Agilent HPLC 1260 (Agilent Technologies, USA) equipped with a diode array detector was applied to analyze the main changes of anthocyanins in native and digested samples. The separation of anthocyanins was performed on a Sepax C18-H column (250 × 4.6 mm inner diameter, with a particle size of 5 μm) and maintained at 25 °C. Two solvents, A, 2.0% formic acid and B, acetonitrile, were used as mobile phases at 0.8 mL/min as the following gradient profile: 0–2 min, 93.0% A; 2–10 min, 80.0% A; 10–40 min, 20.0% A; 40–41 min, 93.0% A; and 41–50 min, 93.0% A. Sample was filtered through 0.45 μm membrane disc filter and injected into the column with a 20 μL volume. The detection was performed at 520 nm. The main anthocyanins in sample were tentatively identified according to the consistency retention time of the authentic anthocyanins standards under the same analysis conditions and based on previous reports (Hou et al., 2010, 2013). All samples were injected in triplicate.

2.6. DPPH radical scavenging assay

The total antioxidant activity of the undigested and digested purple rice anthocyanins was determined according to the DPPH radical scavenging method described earlier (Correa-Betanzo et al., 2014; Jacob et al., 2012) with minor modification. A 0.5 mL of each sample was added into 2.0 mL of 0.1 mM DPPH reagent, and mixed well. Then, all