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Stability of anthocyanins in *bokbunja* (*Rubus occidentalis* L.) under *in vitro* gastrointestinal digestion

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ABSTRACT

The stability of anthocyanins in *bokbunja* (*Rubus occidentalis* L.) extract was investigated using an *in vitro* simulated gastrointestinal digestion. Ethanolic extract from *bokbunja* was digested with pepsin/HCl for 2 h at 37 °C, followed by pancreatin/bile salts for 2 h at 37 °C. Four anthocyanins including cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside and cyanidin-rutinoside were identified in the *bokbunja* extract. The total anthocyanin content of *bokbunja* was 3.76 mg cyanidin-3-glucoside equivalents/g fresh weight (FW). Gastric digestion had no significant effect on anthocyanins. However, intestinal digestion substantially decreased anthocyanins up to 1.70 mg/g FW, corresponding to 45% of that in the *bokbunja* extract. This indicates that about half of anthocyanins can reach an intestinal tract. In addition, a new compound comprised of cyanidin-3-glucosdie, catechin and acetaldehyde in a separated study was observed after *in vitro* gastrointestinal digestion. This shows that anthocyanins could be transformed into other compounds with different biochemical properties.

1. Introduction

Bokbunja (*Rubus occidentalis* L.) is a black raspberry cultivated widely in Korea. It is a rich source of antioxidants, such as anthocyanins, tannins and phenolic acids (Chung and Lim, 2012; Choi & Kwak, 2014; Lee, Dossett, & Finn, 2013). Anthocyanins have drawn much attention recently due to their anti-inflammatory, anti-atherosclerotic and anti-cancer effects (Chung and Lim, 2012; Jeon et al., 2009; Lee, Dossett, & Finn, 2014). Anthocyanins (including cyanidin-3-sambubioside, cyanidin-3-xylosyrutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside) have been found in *bokbunja* (Chung and Lim, 2012; Lee et al., 2013).

Earlier study revealed that about 1% of the anthocyanins individuals consume are present in plasma (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). EI Mohsen et al. (2006) found *p*-hydroxybenzoic acid in rat stomachs after consumption of pelargonidin, suggesting that anthocyanins are unstable in gastrointestinal conditions. Vitaglione et al. (2007) reported that protocatechuic acid is a major metabolite of cyanidin-3-glucoside in humans, which comprises 73% of all anthocyanins ingested by humans. These findings indicate that the stability of anthocyanins in gastrointestinal conditions should be investigated to predict its bioavailability after consumption. Although *in vitro* digestion models are different from human or animal models, they are often used due to their simplicity and low cost. To the best of our knowledge, the *in vitro* digestion of anthocyanins from *bokbunja* has never been reported.

Therefore, the objectives of this study were i) to assess the effect of *in vitro* simulated gastrointestinal digestion on anthocyanins from *bokbunja* and ii) to elucidate the formation pathway of anthocyanin adducts generated from anthocyanins in gastrointestinal conditions.

2. Materials and methods

2.1. Chemicals and materials

Kuromanin chloride (cyanidin-3-glucoside, ≥95%), potassium chloride, sodium acetate, formic acid, hydrochloric acid, sodium bicarbonate, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol and acetonitrile were purchased from Samchun Pure Chemical (Pyeongtaek, Korea). Fully ripened *bokbunja* was purchased from a local farm (Gochang, Korea).

2.2. Extraction of anthocyanins

Bokbunja fruits were freeze-dried at −50 °C at 1.1 Pa pressure for 48 h (EYELA freeze-drier FDU-1200, Tokyo, Japan). The dried fruit (2.0 g) was extracted with 150 mL of 80% (w/w) ethanol containing 0.1% HCl for 1 h at 60 °C using a shaking water bath (JSSB-30T, JS

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Research Inc., Gongju, Korea) at 120 rpm. The mixture was centrifuged (ST16R, Thermo Fisher SCIENTIFIC, Braunschweig, Germany) at 14,981 × *g* for 10 min at 4 °C. The supernatant was put into an amber bottle and the pellet was re-extracted using the same procedure as described above. Combined supernatant was used for simulation and analysis of gastrointestinal digestion *in vitro*.

2.3. In vitro gastrointestinal digestion

In vitro simulated gastrointestinal digestion was performed similarly to previous studies (Liang et al., 2012; Sun, Huang, Cai, Cao, & Han, 2015) with some modifications. The stomach solution was prepared as follows: pepsin from porcine gastric mucosa (12,000 unit/28 mL) was dissolved in 0.1 N HCl solution. The solution was mixed with 21 mL of *bokbunja* extract, which had a pH of 1.3, and then the solution was purged with nitrogen in a shaking water bath at 120 rpm for 2 h at 37 °C under dark conditions. The mixture was added to 2.91 mL of 1 N sodium bicarbonate, which was adjusted to a pH of 5.5. The small intestine solution was prepared as follows: 28 mL of pancreatin from porcine pancreas (4 mg/mL) and 14 mL of bile salts (25 mg/mL) were dissolved in 0.1 N sodium bicarbonate. This was adjusted to a pH of 7.5 by adding 10 μL of 1 N sodium bicarbonate. The mixture was purged with nitrogen and then incubated in a shaking water bath at 120 rpm for 2 h at 37 °C under dark conditions.

2.4. Determination of total anthocyanin

Total anthocyanin content was determined using the pH differential method (Lee, Durst, & Wrolstad, 2005). According to this method, 25 mM potassium chloride and 0.4 M sodium acetate were used as buffer solutions with a pH of 1.0 and 4.5, respectively. The *bokbunja* extract, stomach digestion mixture and small intestine digestion mixture were diluted using the buffer solutions described above. Absorbance was measured at 530 nm and 700 nm using a UV–visible spectrophotometer (Biochrom Libra S22, Santa Barbara, CA, USA). Anthocyanin content was expressed as cyanidin-3-glucoside equivalents:

$$\text{Anthocyanin content (cyanidin}-3-\text{glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where A = pH 1.0 ($A_{530\ nm} - A_{700\ nm}$) − pH 4.5 ($A_{530\ nm} - A_{700\ nm}$); MW = molecular weight of cyanidin-3-glucoside (449.2 g/mol); DF = dilution factor and ε = molar extinction coefficient of cyanidin-3-glucoside (26,900 L/mol × cm).

2.5. Identification of anthocyanins

Chromatographic analysis was performed using a Waters® ACQUITY™ ultra-performance liquid chromatograph (UPLC) system (Waters, Milford, MA, USA). This instrument was equipped with a reverse-phase column (CORTECS™ UPLC® C18 1.6 μm, 2.1 × 50 mm) for chromatographic separation. The injection volume was 5 μL, and the flow rate was 0.2 mL/min. The column was maintained at 40 °C, and the mobile phase consisted of 1% formic acid in distilled water (A) and acetonitrile (B). The gradient was performed as follows: 0–10 min, 95–80% A; 10–12 min, 80–50% A. The UPLC system was coupled with a quadruple-time-of-flight-tandem mass spectrometry (Q-TOF-MS/MS) system (SYNAPT™ G2) equipped with electrospray ionization (ESI), which was set to the positive mode. The capillary voltage was set at 3000 V, and the cone gas was set at 100 L/h. The desolvation gas was set to 800 L/h at a temperature of 350 °C and a source temperature of 120 °C.

2.6. Synthesis of cyanidin-3-glucoside adducts

Cyanidin-3-glucoside, one of the anthocyanins identified in the *bokbunja* extract, reacted with catechin and acetaldehyde under the same conditions used to simulate gastrointestinal digestion of the extract *in vitro*. Cyanidin-3-glucoside was dissolved in 80% ethanol containing 0.1% HCl with a content of 38 μg/mL, which is identical to the concentration in the *bokbunja* extract (Table 2). Catechin and acetaldehyde were separately prepared with the same concentration. The three compounds were mixed and underwent *in vitro* gastrointestinal digestion as described above.

2.7. Quantification of anthocyanins

Anthocyanins were identified using a high-performance liquid chromatograph (HPLC) system (Agilent Technologies, 1260 Infinity, Waldbronn, Germany) equipped with a diode array detector (DAD). The extract was filtered with a 0.45 μm Millex-FH hydrophobic fluoropure (PTFE) membrane (Millipore, Milford, MA, USA). Anthocyanin separation was performed using a Poroshell 120 SB-C_{18} column (2.7 μm, 4.6 × 150 mm i.d., Agilent, Newport, DE, USA) at 25 °C. The injection volume was 10 μL and the flow rate was 0.5 mL/min. The mobile phase consisted of 0.2 M phosphoric acid (solution A) and 0.2 M phosphoric acid with 2:8 acetonitrile (v/v) (solution B). The gradient was as follows: 0–10 min, 100–84% A; 10–25 min, 84–84% A; 25–28 min, 84–20% A; 28–35 min, 20–20% A. The separated anthocyanins were monitored at 530 nm. All cyanidin derivatives were quantified using an authentic cyanidin-3-glucoside.

2.8. Quality assurance/quality control

Blank and calibration samples were used for the determination of a linearity, sensitivity and precision for the HPLC method. A blank was firstly injected in the HPLC instrument to identify a baseline problem and pump pressure, and also check a potential contamination of column, mobile phases and injection port. The standard sample of cyanidin-3-glucoside was repeatedly injected three times to evaluate a peak shape, retention time and peak area. The symmetrical peaks had small variations (± 0.03 min) for retention time. Calibration curve showed a good linear relationship in the range of 0.05–100 μg/mL for cyanidin-3-glucoside ($r^2 \geq 0.999$). A limit of detection (LOD) of cyanidin-3-glucoside, defined as a signal-to-noise ratio of three, was 10 ng/mL. For the precision performed by a triplicate application of each sample, the relative standard deviation of peak areas was 1.29% for cyanidin-3-glucoside. Therefore, this shows that the HPLC analysis is suitable for the analysis of cyanidin-3-glucoside and its derivatives.

2.9. Statistical analysis

All data were presented as mean ± standard deviation. A one-way analysis of variance (one-way ANOVA) was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was performed to determine the statistical differences among different digested samples ($p < 0.05$).

3. Results and discussion

3.1. Total anthocyanin content

The total anthocyanin contents of the *bokbunja* extract and gastrointestinal digestion samples were determined using the pH differential method. As shown in Fig. 1, the total anthocyanin content of the *bokbunja* extract was 19.34 mg cyanidin-3-glucoside equivalents/g dry weight, which corresponded to 3.76 mg/g fresh weight. This is about two fold higher than the content found in previous investigations: 1.85 mg/g fresh weight (Choi & Kwak, 2014) and 0.1–1.8 mg/g fresh