LWT - Food Science and Technology 55 (2014) 645–649

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Thermal characterization of the anthocyanins from black soybean (*Glycine max* L.) exposed to thermogravimetry

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ARTICLE INFO

Article history:
Received 18 August 2011
Received in revised form
24 September 2013
Accepted 2 October 2013

Keywords:
Anthocyanin
Black soybean
Cyanidin-3-glucoside
Degradation
Thermogravimetry

ABSTRACT

One anthocyanin compound, cyanin-3-glucoside, was isolated and identified by high performance liquid chromatography tandem mass spectrometry (HPLC_MS/MS) from seed coat of black soybean variety, Heizi to assess its thermal characterization in vitro. The thermal characterization of anthocyanin was studied by thermogravimetry (TG) analysis. The results from the TG curves showed the decomposition of anthocyanin occurred in three stages. Glucose cleaved from the cyanin-3-glucoside occurred in the first stage. Degradation of cyanin and small amounts of cyanin-3-glucoside appeared mainly in the second stage. The sugar in the anthocyanin was decomposed in the third stage. Thermodynamic analysis was applied by free dynamic model. TG-isothermal experiments for the anthocyanins from black soybean seed coats confirmed the correct of the thermodynamic analysis.

1. Introduction

Black soybean (*Glycine max* L.) accumulates high amounts of anthocyanins, which base with cyanidin, delphinidin, petunidin, and pelargonidin, almost exclusively as 3-*O*-gluscosides in the seed coat (Choung et al., 2001; Kovinich, Saleem, Arnason, & Miki, 2010; Lee et al., 2009). However, the anthocyanin compositions are diverse among different black soybean varieties (Choung et al., 2001; Kuroda & Wada, 1933; Yoshikura & Hamaguchi, 1969). Anthocyanins in black soybean seed coat have antioxidant activities, α-glucosidase inhibition, regulation of adhesion molecules, protection from ischemia, reperfusion heart injury, stimulation wound healing in fibroblasts, and prevention inflammation in endothelial cells (Inagaki, Morimura, Shigematsu, Kida, & Akutagawa, 2005; Kim et al., 2006; Matsui et al., 2001; Nizamutdinova et al., 2009). In addition, they had in vitro antioxidant activity in human low density lipoprotein (Astadi, Astuti, Santoso, & Nugraheni, 2009).

Anthocyanins are unstable compounds which can induce the chemical transformation and affect the performance and biological activity of anthocyanin-containing foods. It was postulated that anthocyanins degraded by two pathways. One is the loss of glycosyl moieties to form aglycone, followed by the formation of a more unstable α-diketone, and then aldehydes and benzoic acid derivatives are formed (Sadilova, Stintzing, & Carle, 2006). Another pathway was postulated that the pseudobase was formed first, followed by the formation of chalcones and coumarin glycosides, and the chalcones would undergo a further degradation. When anthocyanins are exposed to different conditions, they exhibit different degradation mechanisms which induce different degradation route (Zhang, Sun, Hu, & Liao, 2010). However, the degradation mechanism of anthocyanins from black soybean seed coat has not been reported yet.

Many foods are thermally processed prior to consumption and this process can greatly influence the anthocyanin contents in the final products (Giusti & Wrolstad, 2003; Patras, Brunton, Donnell, & Tiwari, 2010). Thermodynamic analysis can give a deep insight into the anthocyanin conversion. Many anthocyanin degradations have been reported to follow the first-order kinetic model based on prediction (Gradinaru, Biliaderis, Kallithraka, Kefalas, & Garcia-Viguera, 2003; Wang & Xu, 2007; Yang, Han, Gu, Fan, & Chen, 2008). However, for simple reactions the evaluation of reaction degree with the *n*-th order model is possible. For complex reactions the function of reaction degree is complicated and not generally known. In such cases the *n*-th order algorithm produces unreasonable kinetic data. However, model free kinetics is based on the theory that reaction degree and the activation energy are constant for a certain value of conversion (iso-conversional method). With model free kinetics, accurate evaluations of complex and simple

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0023-6438/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2013.10.007

reactions can be performed. Therefore, the reaction degree of anthocyanins from black soybean exposed to thermogravimetric apparatus was evaluated with model free kinetics in this research.

In this study, anthocyanin was identified by high performance liquid chromatography tandem mass spectrometry (LC_MS/MS) from black soybean variety, Heizi seed coat. Furthermore, the thermal characterization and degradation mechanism of anthocyanin were studied by thermogravimetric (TG) analysis applied by free dynamic model. TG-isothermal experiments for the anthocyanins from black soybean seed coats were used to confirm that the thermodynamic analysis was suitable. Results would be useful for the application of anthocyanins from black soybean seed coat as colorants or antioxidants in foods.

2. Materials and methods

2.1. Chemicals

Ethanol anhydrous, methanol, acetontrile were purchased from Dima Technologies (Beijing, China). Formic acid (≥98%) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Standard of cyanin-3-glucoside was obtained from Polyphenols Laboratories AS (Sandnes, Norway). These solvents/chemicals used were chromatography grade. Syringe filter units 0.22 μm were supplied by Hercules (Beijing, China). Distilled water was used.

2.2. Extraction and purification of anthocyanins

Black soybean variety, Heizi was supplied by Beijing Academy of Agriculture and Forestry Sciences (Beijing 2011, China). Hand-peeled seed coats were extracted twice with 60% ethanol (pH = 3.0). Anthocyanins extraction conditions were: the volume ratio of black soybean seed coat and extracted solution was 1:15; temperature, 50 °C; extraction time, 1 h. Resultant extraction solution was concentrated with a rotary evaporator (BŰCHI R-215, Flawil, Switzerland) then filtered by a 0.22 μm syringe filter. The extracts were applied on an Amberlite XAD-7 column (60 cm × 1.6 cm, Sigma, Santa clara, USA). After washing the column with 10-fold column volume H_2O contained 0.5 mL/100 mL TFA, the extracted anthocyanins were eluted by methanol containing 0.5 mL/100 mL TFA. The elution was evaporated.

Then 5 mL concentrated isolated methanol pigments were fractioned by Sephadex LH-20 chromatograph (60 cm × 1.6 cm, GE, Fairfield, USA) using H_2O (0.5 mL/100 mL TFA)-MeOH (7:3) as eluent. The flow rate was 1.0 mL/min. Fractions were collected by a fraction auto-collector (GE, Fairfield, USA). The fractions were evaporated on a rotary evaporator to remove methanol to facilitate the removal of water remaining in the sample. The evaporation temperature was less than 45 °C. The removal of water was carried out on a freeze drier (LGJ-10, Beijing Songyuan Huaxing Technology Developing Co., Beijing, China). The homogeneity of individual fraction was checked by analytical HPLC-DAD. Then they resolved in distilled water containing 0.5 mL/100 mL formic acid and filtered through a 0.22 μm syringe filter for HPLC_MS/MS analysis and TG experiment.

2.3. HPLC_MS/MS analysis

The HPLC_MS/MS analysis of purified anthocyanins were performed by an Agilent 1200 series liquid chromatograph containing an autosampler coupled to a 6300 series ion trap mass spectrometer (Agilent, Santa Clara, USA). 3 μL of the sample was injected onto an analytical scale Zorbax SB-AO column (particle size, 1.8 μm; 100 × 3.0 mm, Agilent, Santa Clara, USA) maintained at 25 °C. The elution mode was a linear acetonitrile gradient (10%–60%, 30 min) containing 0.5% formic acid at a flow rate of 0.3 mL/min for HPLC_MS/MS analysis.

DAD detection at 280 and 520 nm were performed. Anthocyanins were detected using ion trap in the positive ion mode. Used MS parameters were as follows: nebulizing pressure, 30 psi; source temperature, 110 °C; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min nitrogen; scan ranger, m/z 100–1500; smart parameter setting, compound stability, 20%; trap drive level, 100%. Identification of anthocyanidin compound was based on the retention time and LC/MS m/z values with reference to cyanin-3-glucoside standard.

2.4. Thermal characterization of anthocyanins from black soybean seed coat

Thermodynamic characterization of the sample was measured by a Mettler Toledo thermogravimetry (Zürich, Switzerland), over a temperature interval of 30–900 °C at heating rate of 5, 10, 15 °C/min, respectively. The thermograph was analyzed by the stare software (version 9.30). The thermodynamic parameter and characterization of the sample was studied. Especially, the degree of conversion (α) of the sample with time and its relationship with temperature were studied. To confirm the accuracy of dynamic analysis by model free kinetics, the TG-isothermal curves were obtained over 10 min at temperatures of 170, 200, 210, 250 °C, which the temperatures of joints between the first and second stage of anthocyanin decomposition, as observed in the TG dynamic profiles.

3. Results and discussion

3.1. Purification and identification of anthocyanins from black soybean seed coat

Anthocyanins were extracted from black soybean seed coats with 60% ethanol. There was only one peak in HPLC chromatogram of the crude extract detected at 520 nm, while several peaks at 280 nm (data not shown), which indicating that resultant solution was a mixture containing one kind of anthocyanin, phenolic acids, sugars, proteins, and other flavonoids. Amberlite XAD-7 was chosen to separate the compounds above. Sugars and proteins have no affinity for the resin and can be washed away by water with 0.5 mL/100 mL TFA. The compounds are continuously isolated using Shephdex LH-20, which remove the phenolic acids and other flavonoids. Anthocyanins, which are the target compounds, were collected and identified by high-performance liquid chromatography (HPLC). 6 mg purified anthocyanin was obtained from 100 g black soybean seed coat.

HPLC chromatogram of the anthocyanin fraction at 520 and 280 nm as a detection wavelength was shown in Fig. 1A. There was only one peak with a retention time of 8.1 min in Fig. 1A. Furthermore, mass spectrometry was used to detect the molecular mass and to identify the structure. A single prominent protonated molecular ion peak was found at m/z 449 $[M + H]^+$ in the MS spectrum, indicating only one compound in peak (Fig. 1B). Further results (Fig. 1C) were found by using MS/MS. One fragment ion (m/z 287) was found in MS/MS spectrum, produced through losing one group with mass of 787–625 = 162 from parent ion, indicating sugar moiety as the lost fragment. Finally, peak 1 was identified as cyanin-3-glucoside according to their mass spectra, previous research and the reference of standard (Islam, Yoshimoto, & Terahara, 2002; Truong et al., 2010). UV/visible spectra of this peak performed by a diode array detector were characterized between 200 and 800 nm (data not shown), which confirming that the compound was cyanin-3-glucoside. According to the results above, there was only one