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Depolymerisation optimisation of cranberry procyanidins and transport of resultant oligomers on monolayers of human intestinal epithelial Caco-2 cells

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Procyanidins in cranberries are predominantly polymers (>85%). The objective of this study was to optimise the depolymerisation of polymers and to investigate the absorption of resultant oligomers on Caco-2 cell monolayers. Depolymerisation conditions were optimised using response surface methodology. Depolymerisation, with or without added epicatechin, yielded 644 μg and 202 μg of oligomers (monomer through tetramers) per mg of partially purified polymers (PP), respectively. Oligomers (yielded from both methods) were transported through Caco-2 cell monolayer despite absorption rates being low. With the aid of response surface methodology, the optimum depolymerisation conditions were determined to be 60 °C, 0.1 M HCl in methanol and 3 h without added epicatechin. The predicted maximum yield was 364 μg oligomers per mg of PP. The optimum depolymerisation condition with added epicatechin shared the same temperature, acid concentration and reaction time, in addition to an epicatechin/PP mass ratio of 2.19. Its predicted maximum oligomer yield was 1089 μg/mg. The predicted yields were verified by experimental data.

1. Introduction

Procyanidins in cranberries consist of oligomers and polymers of different sizes (Gu et al., 2003). Oligomers refer to procyanidin monomers through tetramers. Polymers include pentamers and above. A-type oligomeric procyanidins from cranberries were known to inhibit the adherence of uropathogenic *Escherichia coli.* Whereas B-type were not (Foo, Lu, Howell, & Vorsa, 2000; Shoji et al., 2005; Sugiyama et al., 2007). Procyanidins (dimers through tetramers) were absorbable *in vitro* (Ou, Percival, Zou, Khoo, & Gu, 2012; Zumdick, Deters, & Hensel, 2012) and *in vivo* (Baba, Osakabe, Natsume, & Terao, 2002; Stoupi et al., 2010), whereas larger procyanidins are not absorbable. In cranberry, however, these absorbable oligomers only account for 15% of total procyanidins, while the rest are polymers (Gu et al., 2002). Such composition limits both the bioavailability and bioactivities of procyanidins in cranberries.

Several methods were explored to convert polymers into absorbable oligomers and monomers. Extrusion of sorghum increased the levels of procyanidin oligomers with degree of polymerisation (DP) $\leqslant 4$, while it decreased the amount of polymers with DP $\geqslant 6$ (Awika, Dykes, Gu, Rooney, & Prior, 2003). Extrusion also increased procyanidin monomers and dimers in blueberry pomace (Khanal, Howard, Brownmiller, & Prior, 2009). The improvement of procyanidin bioavailability through extrusion was verified in weanling pigs (Gu, House, Rooney, & Prior, 2008). Our recent study applied flavan-3-ols as chain breakers to depolymerize procyanidins under a mild condition (Liu, Zou, Gao, & Gu, 2013). In this method, H^+ catalyzes the cleavage of the interflavan bonds to form carbocation at C4. A nucleophilic addition between carbocations and added flavan-3-ols resulted in the generation of new oligomers. The chemical structure of newly generated oligomer after depolymerisation was similar to that of naturally occurring procyanidins. However, it was not known if these oligomers were absorbable. Because bioactivities of procyanidins largely depend on their bioavailability, it is important to investigate their absorption. Therefore, the objective of this study was to investigate the transport of depolymerized cranberry procyanidins on Caco-2 cell monolayers. If they can be absorbed, the depolymerisation process can be optimised using response surface methodology.

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2. Materials and methods

2.1. Chemicals and materials

Freeze-dried cranberries were provided by Ocean Spray Cranberries, Inc (Lakeville-Middleboro, MA). (−)-Epicatechin and Sephadex LH-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Amberlite FPX 66 resin was a product from the Dow Company (Midland, Michigan). Caco-2 cells, originating from human colorectal carcinoma, were obtained from the American Type Culture Collection (Manassas, VA). Other reagents were obtained from the Fisher Scientific Co. (Pittsburg, PA).

2.2. Preparation of partially purified procyanidin polymers (PP)

Five hundred grammes of dried cranberry powder was extracted into 4 L of methanol at room temperature for 48 h. Extracts obtained after vacuum filtration were combined and concentrated under partial vacuum using a rotary evaporator. The concentrated extract was re-suspended in 100 ml of water and loaded onto a column (3.8 × 44 cm) packed with Amberlite FPX 66 resin. The column was eluted with 5 L of de-ionised water to remove sugars, followed by 2 L of methanol, to yield cranberry phytochemical powder (about 28 g). Part of this powder (27 g) was suspended in 80 ml of 30% methanol and loaded onto a column (28 cm, 5.8 cm i.d.) packed with Sephadex LH-20, which was soaked in 30% methanol for over 4 h before use. The column was eluted with 30% methanol (1.6 L), 60% methanol (1.2 L), 80% methanol (1.2 L), 100% methanol (1.2 L) and 70% acetone (1.2 L). Every 400 ml of effluent was collected as a fraction. The second fraction of the 70% acetone elution was dried and stored for depolymerisation experiments. The profile of procyanidins in this extract was analysed using HPLC–MS^n.

2.3. Depolymerisation of cranberry procyanidins

Depolymerisation was carried out, with or without added epicatechin. In experiments without added epicatechin, cranberry procyanidin extract was dissolved in methanol to have a stock solution with a concentration of 10 mg/ml. The stock solution was then mixed with an equal volume of 2 M methanolic HCl. In the depolymerisation with added epicatechin, procyanidin extract and (−)-epicatechin were dissolved in methanol to achieve a concentration of 20 mg/ml. Afterwards, equal volumes of extract and (−)-epicatechin were mixed with 2 volumes of 2 M methanolic HCl. The final concentration of cranberry procyanidin extract was 5 mg/ml. Depolymerisations were carried out in a 60 °C water bath for 60 min for both methods. The reaction was stopped by adjusting the pH of the mixture to 5 with 1 M $NaHCO_3$. The solutions were dried in a SpeedVac concentrator at 45 °C. Then, the dried sample was dissolved in water and partitioned with ethyl acetate 3 times. The ethyl acetate fraction was collected and dried in a SpeedVac concentrator.

2.4. HPLC–ESI-MS^n analysis of procyanidins

Chromatographic analyses were performed on an Agilent 1200 HPLC system (Palo Alto, CA) equipped with a binary pump, an autosampler, a fluorescence detector, and a high capacity (HCT) ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Separation was carried out on a Luna Silica (2) column (250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA) at a column temperature of 37 °C. The binary mobile phase consisted of (A) methylene chloride/methanol/acetic acid/water (82:14:2:2, v/v/v/v) and (B) methanol/acetic acid/water (96:2:2, v/v/v). The 70 min gradient was as follows: 0–20 min, 0.0–11.7% B linear; 20–50 min, 11.7–25.6% B linear; 50–55 min, 25.6–87.8% B linear; 55–65 min, 87.8% B isocratic; 65–70 min, 87.8–0.0% B linear; followed by 5 min of column re-equilibration before the next injection. Excitation and emission of the fluorescent detector were set at 231 and 320 nm, respectively (Robbins et al., 2009). Electrospray ionisation, in negative mode, was performed using a nebulizer at 50 psi, drying gas 10 L/min, and drying temperature 350 °C, capillary 4000 V. Mass spectra were recorded in a range of m/z 150–2200. The most abundant ion in full scan was isolated, and its production ion spectra were recorded. A calibration curve was generated using (−)-epicatechin standard. Procyanidins were estimated using relative response factors calculated according to Robbins et al. (2012). The relative response factor of nonamers was employed to estimate the amount of polymers. The yield of depolymerisation was defined as the amount of oligomers produced per mg of partially purified polymers.

2.5. Transport experiments

Transport experiments were carried out, following the procedures described in a previous study (Ou et al., 2012). The concentration of depolymerized samples in Hank's Balanced Salt Solution (0.5% DMSO) was equivalent to 2 mM epicatechin.

2.6. Optimisation of depolymerisation conditions

A central-composite experimental design was utilised to study the response pattern and determine the optimum conditions. In depolymerisation without added epicatechin, variables included time (ranged from 20 to 180 min), temperature (ranged from 25 to 60 °C), and acid concentration (ranged from 0.1–2 M HCl in methanol). In the method with added epicatechin, an additional factor of epicatechin/polymer ratio (w/w) (ranged from 0 to 3) was taken into consideration. In total, 20 experiments for depolymerisation without added epicatechin and 30 for depolymerisation with added epicatechin approach were conducted according to the design. Duplicates were performed for each run. Six replicates at the centre of the design were used to allow the pure error square sum estimation. The conditions at centre point were 100 min, 42.5 °C, 1.05 M HCl in methanol for depolymerisation without added epicatechin. The centre point for depolymerisation with added epicatechin had the same time, temperature and HCl concentration, while the epicatechin/polymer ratio was 1.5. Experiments were randomized to minimize the effects of unexplained variability in the responses obtained due to external factors.

Experimental data were analysed using JMP software (Version 10.0), SAS Institute Inc., Cary, NC). Quadratic polynomial regression models were proposed to predict the Y variable-yield of oligomers. The model proposed for the response of the Y fitted equation was as follows:

$$Y_1 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \varepsilon \quad (1)$$

$$Y_2 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + \varepsilon \quad (2)$$

where Y_1 and Y_2 are the response (yield of oligomers) for depolymerisation with or without added epicatechin, respectively. The coefficients of the polynomial were represented by b_0 (constant term), b_1, b_2, b_3 and b_4 (linear effects), b_{11}, b_{22}, b_{33}, and b_{44} (quadratic effects), b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, and b_{34} (interaction effects) and ε (random error). The fitted polynomial equations were