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In vitro bioaccessibility and physicochemical properties of phytosterol linoleic ester synthesized from soybean sterol and linoleic acid

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ABSTRACT

Phytosterols are bioactive components capable of reducing cholesterol level in serum and reducing risk of arteriosclerosis. In this study, conditions for the synthesis of maximum yield of phytosterol linoleic ester (PLE) was optimized and the physicochemical properties and *in vitro* bioaccessibility of the PLE were assessed. Under the optimized condition of 1:1.1 mol ratio of phytosterol and linoleoyl chloride at 80 °C for 1.5 h, the conversion rate of phytosterol reached 96.1%. Its solubility in oil increased 20 times, up to 33.8%. Also, peroxide value of PLE was much lower than linoleic acid (32.9 and 47.0 mmol/kg), which means better oxidative stability. Bioaccessibility of PLE was affected by time, concentration of bile extract, and dissolved medium. It was 4.93% alone, increased by 2.5 times compare to phytosterol; or 53.46% in oil, under the condition of 40 mg/mL bile extract for 120 min. In conclusion, under the tested condition, phytosterol conversion rate, its solubility in oil and bioaccessibility were improved significantly. The method showed great potential in manufacture high quality and quantity of PLE.

1. Introduction

Phytosterols are bioactive compounds in plant, and also integral components of oil unsaponifiable matter. The composition and content of phytosterols (PS) differ in different vegetable oils, with the most important being β-sitosterol, stigmasterol, campesterol and brassicasterol (Moreau, Whitaker, & Hicks, 2002; Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). Studies have shown the importance of PS in reducing cholesterol levels in the serum (Brufau, Canela, & Rafecas, 2008; Wolfs, de Jong, Ocké, Verhagen, & Monique Verschuren, 2006). Linoleic acid (LA) is a common polyunsaturated fatty acid with large amounts found in safflower seed oil, sunflower oil, walnut oil and soybean oil. LA is an essential fatty acid and can also reduce the risk of arteriosclerosis in animal model and humans. Both PS and LA are regarded essential molecules since they cannot be synthesized in the human body. These molecules must be obtained from food sources.

Phytosterols are insoluble in water and their solubility in oil is just about 1% (Yang, Oyeyinka, & Ma, 2016). This trait limits their wide application in food/pharmaceutical industry. In order to enhance the application and improve the bioaccessibility of PS, researchers have utilized the esterification method to produce phytosterol esters from PS and fatty acids. Chemical synthesis and biological synthesis are the two main methods currently employed. Chemical synthesis has several advantages including providing good conversion rate (CR), and results in high productivity. However, it has several drawbacks too. For example, chemical esterification requires the use of catalysts such as magnesium oxide, lanthanum oxide, zinc oxide, aluminum oxide, and aluminum triiodide (Hang & Dussault, 2010; Meng, Pan, & Yang, 2010; Robles-Manuel, Barrault, & Valange, 2011; Valange et al., 2007). The major challenge is the difficulty in separating catalyst from the final product. Another major challenge is the high temperature used during the synthesis, which may lead to the production of by-product. Biological synthesis uses a relatively low temperature, produces no or less by-product, but takes a longer time with low CR products (Villeneuve et al., 2005; Vu, Shin, Lim, & Lee, 2004).

Recently, we synthesized PS esters using PS from soybean and acetic anhydride (Yang et al., 2016). The optimum condition for the production of high yield of PS ester (99.4%) was found to be a temperature of 135 °C for 1.5 h with a mole ratio 1:1 for phytosterol and acetic anhydride, respectively. Furthermore, Fourier transform infrared spectroscopic and gas chromatography-mass spectrometric studies revealed that no other harmful by-products were formed during the process

Abbreviations: PS, phytosterol; LA, linoleic acid; PLE, phytosterol linoleic ester; CR, conversion rate; LC, linoleyl chloride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid

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(Yang et al., 2016). With the growing interest in the synthesis of high-quality PS ester products using new technology, it may be necessary to investigate promising alternatives to the traditional chemical methods. Hence, in this paper, PLE was first synthesized from soybean sterol and LA using acyl chloride method in order to optimize reaction conditions. The physicochemical properties and *in vitro* bioaccessibility of the PLE were thereafter assessed.

2. Materials and methods

2.1. Materials

Linoleic acid (≥99%), trypsin, pepsin, sodium taurocholate, as well as lipase (Type II), colipase and cholesterol esterase from bovine pancreas were purchased from Sigma-Aldrich company (America). Acetone and acetonitrile used were of chromatography grade. Hexane, PCl_3, NaOH, $NaHCO_3$, NaCl, $CaCl_2$, HCl, KH_2PO_4 were analytical grade. Soybean sterol (≥95%, seperated and purified from soybean oil deodorized distillate), soybean oil, rapeseed oil, peanut oil, corn oil, sunflower oil were obtained from Jiusan Grains & Oils Industries Group Co., Ltd (China). Standards of campesterol (≥98%), stigmasterol (≥98%), β-sitosterol (≥98%) were purchased from Chengdu Purification Technology Development Co., Ltd (China). Components of soybean sterol were analyzed by using a GC (7890A, Agilent, USA), the soybean sterol contents were β-sitosterol; 46.7%, stigmasterol; 27.4% and campesterol; 25.3%.

2.2. Preparation of linoleyl chloride

LA was reacted with phosphorus trichloride (PCl_3) as shown in following Equation (1).

$$3C_{17}H_{31}COOH + PCl_3 \rightarrow 3C_{17}H_{31}COCl + H_3PO_3 \quad (1)$$

PCl_3 is a kind of colorless liquid with pungent smell, it has a melting point of −112 °C and boiling point of 76 °C. So, the reaction temperature should not be too high. The mole ratio of LA to PCl_3 used in the reaction was 3:1. LA was put into a reaction bottle which connected to a condenser device. PCl_3 was then transferred slowly into the bottle at room temperature (25 °C), and the solution was constantly stirred. The resulting mixture was kept at a constant temperature of 60 °C for 3 h. Then, the lower layer of H_3PO_3 was separated, and crude linoleoyl chloride (LC) was obtained. The crude product was exposed to vacuum at 65 °C for 0.5 h so that the residual PCl_3 could be removed by distillation.

2.3. Synthesis and purification of PLE

PS was esterified with LC as shown in Equation (2).

$$C_{17}H_{31}COCl + R\text{–}OH \rightarrow C_{17}H_{31}COOR + HCl \quad (2)$$

R-OH represents β-sitosterol, stigmasterol, and campesterol respectively in the equation.

Because the reaction rate of acetylation is fast, the temperature does not need to be too high. PS was reacted with LC in different mole ratio of 1:1, 1:1.1, 1:1.2, 1:1.3, and 1:1.4 respectively. The mixture was reacted at different temperatures (90, 80, 70, 60, and 50 °C) and at different time (0.5, 1, 1.5, 2, and 2.5 h). The generated HCl gas was absorbed by a diluted NaOH solution to promote the reaction. Crude product of PLE was dissolved in hexane and excessive saturated $NaHCO_3$ solution was added into the hexane and mixed thoroughly to remove the non-reacted LC. Finally, the upper layer of hexane was collected and distilled in vacuum to remove hexane, and product of PLE was obtained.

2.4. Analysis of the conversion rate

2.4.1. Preparation of PLE standards

There are no commercial standards of PLE. LC, campesterol (≥98%), stigmasterol (≥98%), and β-sitosterol (≥98%) were used to synthesis standards of PLE at 80 °C for 1.5 h. Mole ratio of PS and LC was 1: 1.1. The crude standards of campesterol linoleic ester, stigmasterol linoleic ester, β-sitosterol linoleic ester were dissolved in hexane respectively. Excessive saturated $NaHCO_3$ solution was added into hexane and mixed thoroughly. Then the hexane layer was taken out and dried with nitrogen gas flow. Aliquot of sample was analyzed by HPLC (2695, Waters, USA) using area normalization method, and a HPLC condition was used (see 2.4.2 section). A noted insignificant amount of PS in the product (PLE ≥98%), connoted that the standard was qualified.

Standards of campesterol linoleic ester, stigmasterol linoleic ester, β-sitosterol linoleic ester were weighed accurately and dissolved in acetone to make a standard solution. 10 μL of five different concentrations (0.01, 0.05, 0.25, 0.5, 1.0 mg/mL) of the standard solution were used to generate a standard curve.

2.4.2. HPLC analysis

Conversion rate (CR) analysis of PS ester was performed with HPLC (2695, Waters, USA) equipped with an ultraviolet detector (UV, 2489, Waters, USA). Mobile phase was acetonitrile and acetone (1:3, V/V), flow rate was 1.0 mL/min. Chromatography column was Symmetry-C18 (4.6 mm × 150 mm, 5 μm) and the detection wavelength was 210 nm. CR was calculated as Equation (3).

$$CR = \left(\frac{\text{Mass of PLE}}{\text{Mass of PS} + \text{Mass of PLE}}\right) \times 100\% \quad (3)$$

2.5. Structural and physicochemical properties

2.5.1. Fourier transform infrared spectrometer (FTIR) analysis

Attenuated total reflectance (ATR) analysis was performed using a FTIR (Cary 630, Agilent, USA) for spectra measurement in the frequency range of 4000-650 cm^{-1}.

2.5.2. Thermodynamic analysis

Differential scanning calorimeter (DSC 1, Mettler-Toledo, Switzerland) was used to determine the thermodynamic properties of the PLE. Sample mass of PLE was 7.3 mg (PS was 5.1 mg). The PLE sample was heated from −20 to 90 °C (PS was from 0 to 180 °C) and then cooled from 90 to −20 °C (PS was from 180 to 0 °C) using a programmed temperature of 10 °C/min. Flow of nitrogen gas was 50 mL/min.

2.5.3. Solubility in oil

The solubility of the synthesized PLE was assessed using previously described method except that mixture of PLE and oil was stirred evenly at 80 °C (Yang et al., 2016). Briefly, excessive PLE was taken and added into soybean oil, rapeseed oil, peanut oil, corn oil, sunflower oil respectively. The mixture of PLE and oil was then stored at −5, 5, 15, and 25 °C until the oil became clear and transparent. The upper layer of oil was taken out. PS content was analyzed by applying GC method as previously described (Naeemi, Ahmad, Alsharrah, & Behbahani, 1995).

2.5.4. pH stability

pH stability was studied at different pH values (2.0–12.0) for the PLE application in food. 300 mL water was taken and divided into six portions (each 50 mL), solution of HCl and NaOH were used to adjust the pH value to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0. 200 mg PLE was added into each of the mixtures and stirred for 30 min at room temperature (25 °C). 20 mL hexane was added into the mixture and the resulting