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Enzymatic synthesis of phytosteryl lipoate and its antioxidant properties

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ABSTRACT

In this work, an enzymatic route for synthesizing phytosteryl lipoate was successfully set up for the first time. The structure of final product phytosteryl lipoate was determined by Fourier Transform Infrared (FTIR), Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR). The maximum conversion of 71.2% was achieved when the following conditions were employed: 150 mmol/L phytosterol, 1: 2.5 M ratio of phytosterol to lipoic acid, 10 g/L of 4 Å molecular sieves and 60 g/L Candida rugosa in 2-methyl-2-butanol/*n*-hexane (1:1, v/v) at 55 °C for 96 h. The physicochemical properties including solubility and antioxidant ability of phytosteryl lipoate in oil were assessed. The results revealed that phytosteryl lipoate possessed over twice as much oil solubility as free phytosterol and also showed better antioxidant ability. Investigation on its biological functions will be the main object in the future work.

1. Introduction

Phytosterol plays important roles in pharmaceutical industries because of the vast physiological significances such as cholesterol-lowering, antioxidant and anticancer functions (Alappat, Valerio, & Awad, 2010; Bard, Paillard, & Lecerf, 2015; Danesi, Gomez-Caravaca, de Biase, Verardo, & Bordoni, 2016; Woyengo, Ramprasath, & Jones, 2009). In addition, plant sterols are one of healthy factors in food (Baumgartner, Mensink, & Plat, 2016; Dong et al., 2016; te Velde et al., 2015). However, the practical application of phytosterol is limited due to its poor solubility in both water and oil, which is caused by its special chemical structure. Consequently, there is a need to modify phytosterol structure with its functions retained. Up to now, most studies have focused on the esterification of phytosterol with fatty acids to improve its solubility, however, there are a few reports on phytosteryl esters with their improved biological functions (Fu et al., 2014; Tan & Shahidi, 2012, 2013). What is noteworthy is that these phytosteryl esters can be decomposed into two initial reactants after digestion (Carden, Hang, Dussault, & Carr, 2015; Lubinus, Barnsteiner, Skurk, Hauner, & Engel, 2013). As a result, it's crucial to choose a suitable reactant which can improve respectively the physicochemical properties and can also perform their beneficial functions together.

Antioxidants have been always attracting the attention of many researchers, since they can prevent not only the oxidation of fat rich food to prolong their storage time but also senescence and some other diseases caused by reactive oxygen species (ROS) and free radicals in body, such as Alzheimer's disease (Rosini et al., 2005). Lipoic acid is a very important kind of vitamin B compounds and mainly presents in the liver tissue of animals and some plants such as tomato, broccoli, carrot and spinach. The high electron density in closed five-membered ring imparts lipoic acid significant electrophilic property and the ability to react with free radicals. In the body, lipoic acid can be reduced into dihydrogen lipoic acid which has two sulfydryls. Both lipoic acid and dihydrogen lipoic acid have good resistance to oxidation and can work together in fat soluble and water soluble environment. In this relation, they are widely known as natural universal antioxidants. Lipoic acid owns various useful functions for human health, which has been already used in medical treatment, such as the prevention of diabetes, cancer, cardiovascular disease and inflammation (Benchekroun et al., 2016; Derosa, D'Angelo, Romano, & Maffioli, 2016; Moura, de Andrade, dos Santos, & Goulart, 2015; Tsuji-Naito, Ishikura, Akagawa, & Saeki, 2010; Ying et al., 2010). Meanwhile, it is also extensively applied in cosmetics and health food fields (Leysen & Aerts, 2016; Merry, Kirk, & Goyns, 2008). Researchers have already reported the synthesis of lipoic acid conjugates for health applications (Hsieh et al., 2015; Kaki, Balakrishna, & Prasad, 2014; Lahiani, Hidmi, Katzhendler, Yavin, & Lazarovici, 2016). Recent study has demonstrated that the phytosterol/lipoic acid combination could work together and played a preferable and superior cholesterol-lowering role than phytosterol or lipoic acid alone (Rideout et al., 2016). Phytosteryl lipoate as shown in Fig. 1, synthesized through esterification of phytosterol with lipoic acid, was supposed to play better physiological functions with smaller

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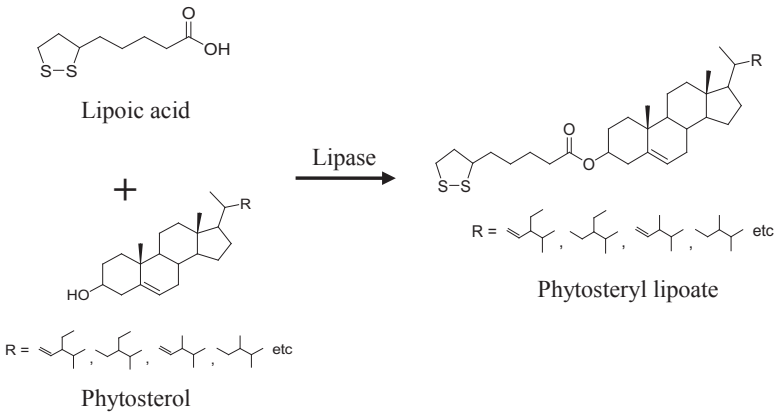

Fig. 1. Synthetic route of phytosteryl lipoate.

amounts than either phytosterol or lipoic acid.

Enzymatic method is a great route for mild reaction, high safety and less by-products, which meets the requirements for good health and green. In recent years, lipases have been widely used for catalyzing esterification reactions of phytosterols. Schär and Nyström produced steryl ferulates using enzymatic method for the first time (Schär & Nyström, 2016). Miao et al. have selected Candida rugosa as catalyst to develop phytosteryl laurate in non-aqueous media (Miao et al., 2014). To the best of our knowledge, the enzymatic synthesis of phytosteryl lipoate has not still been well documented.

The present work was therefore aimed to explore a lipase-catalyzed method to synthesize phytosteryl lipoate and investigate its optimal reaction conditions. After purification by Thin-Layer Chromatography (TLC) and silica gel column chromatography, final product's chemical structure was determined by FT-IR, NMR and MS. Meanwhile, the solubility and antioxidant activity of phytosteryl lipoate were also evaluated.

2. Materials and methods

2.1. Chemicals

Phytosterols were a generous gift from Jiangsu Spring Fruit Biological Products Co., Ltd. (Taixing, P.R. China). The purity of plant sterols was > 97% (63% β-sitosterol and 37% stigmasterol). Lipoic acid was purchased from Xi'an Jinheng Chemical Co., Ltd. (Shanxi, China). Novozym 435 (lipase B from *Candida antarctica*, immobilized on a macroporous acrylic resin, 10,000 PLU/g), Lipozyme TL IM (lipase from Thermomyces lanuginosus, immobilized on silica granulation, 250 IUN/g), Lipozyme RM IM (lipase from Rhizomucor miehei, immobilized on an anionic exchange resin, 275 IUN/g) were obtained from Novo Nordisk Co., Ltd. (Shanghai, China). Candida rugosa (lyophilized powder, Type VII, 847 U/mg) was supplied by Sigma-Aldrich Co., Ltd. (Shanghai, China). Methanol of spectral grade was obtained from Tedia Company Inc. (Shanghai, China). The rapeseed oil was purchased from the local supermarket. The 3 Å and 4 Å molecular sieves and other common reagents of analytical grade were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Enzymatic synthesis of phytosteryl lipoate

Typically, phytosterol (1 mmol) and lipoic acid (1–4 mmol) were dissolved in 10 mL organic solvent at first, followed by the addition of 0–0.4 g of molecular sieves and lipase (0.4–0.6 g). The reaction was carried in a shaking water bath at 150 r/min, for 48–120 h at 40–60 °C. All samples were performed in triplicate.

2.3. Purification of esterified reaction mixture

After the esterification reaction, the mixture was filtered to remove the lipase and molecular sieves, and then the extract was treated with 0.1 M $NaHCO_3$ aqueous solution to neutralize excess lipoic acid. The filtrates were evaporated by rotary evaporator to remove the solvents, then, 1.0 g of the dried sample was dissolved in petroleum ether (60–90 °C)/ethyl acetate/formic acid mixture (15:1:0.02, v/v/v). The obtained solutions were subsequently applied to a silica gel column (24 × 1200 mm) and eluted with petroleum ether (60–90 °C)/ethyl acetate/formic acid (15:1:0.02, v/v/v) at a flow rate of 0.5 mL/min. The eluate was detected by HPLC and collected at the same time, the esters were then obtained after the solvents evaporation by rotary evaporation.

2.4. HPLC analysis

The samples (10 μL) taken out from reaction systems were injected in Waters 1525 HPLC analysis equipped with a symmetry-C_{18} column (5 μm, 4.6 × 150 mm, Waters, USA) at 45 °C. The sample was eluted with methanol (mobile phase) at a flow rate of 1 mL/min. The effluent was monitored with a Grace Alltech 3300 evaporative light scattering detector (ELSD) at 55 °C and nitrogen was used as the carrier gas at a flow rate of 1.5 L/min.

The esterification rate of phytosterol with lipoic acid to form phytosteryl lipoate was calculated via calibration curve and purified phytosteryl lipoate was used as external standard. The conversion was defined as follows:

$$X_{PSE}\ (\%) = C_{PSE}/C_{PS} \times 100 \quad (1)$$

where C_{PSE} is the actual concentration of phytosteryl lipoate at the end of the reaction, C_{PS} is the initial concentration of phytosterol.

2.5. FTIR analysis

The purified phytosteryl lipoate was dried completely under vacuum and then mixed with KBr. FTIR analysis was conducted on a FT-IR spectrophotometer (Thermo Nicolet iS10 FTIR, Thermo Electron, USA) using an attenuated total reflectance method with the spectral scanning scope for 400–4000 cm^{-1}, the number of scans is 32 and resolution is 4 cm^{-1}.

2.6. MS analysis

Liquid chromatography mass spectrometry (Maldi Synapt Q-Tof, Waters, USA) was used to further identify the stigmasteryl ester under positive-ion electron spray ionization (ESI) mode. The MS parameters were listed as follows: capillary voltage, 3.5 kV; cone voltage, 30 V; source block temperature, 100 °C; desolvation temperature, 400 °C;