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Integration of (+)-catechin and β-sitosterol to achieve excellent radical-scavenging activity in emulsions

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ABSTRACT

Novel amphiphilic antioxidant, (+)-catechin-β-sitosterol (CS), was designed and successfully prepared with integration of (+)-catechin and β-sitosterol through the linkage of succinic acid. Sequential esterification was carried out to connect (+)-catechin and β-sitosterol. The identity of CS was confirmed by NMR, IR and MS spectroscopies. DSC analysis revealed that ΔH of CS was much lower than those of (+)-catechin and β-sitosterol, indicating ameliorated crystallinity. The logP measurement demonstrated significantly increased lipophilicity. Then excellent antioxidant activities of the novel antioxidant in typical polyunsaturated lipid O/W and W/O emulsions were unveiled applying β-sitosterol bleaching assay and 5-dodecanoylaminofluorescein (DAF) fluorescent probe method. The antioxidative behavior of CS in emulsion was beyond the polar paradox hypothesis and could be rationalized by effective accumulation at oil/water interface owing to its amphiphilic nature. This study offers a promising solution for development of naturally derived amphiphilic antioxidants for lipid-based systems.

1. Introduction

Inhibition of lipid oxidation by antioxidants to extend the shelf life of food products is essential in food industry. As natural polyphenolic antioxidants, catechins are ubiquitously spread in human diet such as tea, apple, grape and cocoa (Liu, Lu, Kan, Wen, & Jin, 2014; Morina, Takahama, Mojovic, Popovic-Bijelic, & Veljovic-Jovanovic, 2016), and have health beneficial effects including antioxidative, anti-inflammatory, lipid-lowering, antidiabetic and cardiovascular disease prevention activities (Wang, 2011; Yang, Kotani, Arai, & Kusu, 2001; Zaveri, 2006). However, owing to the hydrophilic nature catechins can scarcely dissolve in lipids, which largely limits their application in oxidation sensitive lipid-based systems. Modification by acylation have been extensively attempted to ameliorate the physicochemical properties of catechins due to effectivity and operational simplicity. Usually acylation of catechins was nonspecific and often decreased the antioxidant activity. As a result, we initiated studies on specific modification of catechins to finely tune the physicochemical properties without loss of activities. In our previous study, specific modification of the aliphatic hydroxyl group in C-ring by lipophilic fatty acids was successfully achieved and greatly improved the solubility in lipids with minimal interference of the antioxidant activity of catechins (Hong & Liu, 2016). Therefore, specific acylation of catechins with appropriate lipophilic molecules can effectively modulate their properties.

Other than fatty acids, phytosterols are also valuable lipophilic compounds. Phytosterols are essential triterpene derivatives that are vital structural components of plant cell membranes (Moreau, Whitaker, & Hicks, 2002). Recently, phytosterols have received great attention in food industry mainly because of their capability of lowering both total and LDL cholesterol levels (Laos et al., 2014; Shang, Li, & Zhang, 2015). Hence, we reasoned that phytosterols could be excellent lipophilic partners to modulate physicochemical properties of catechins. The combination of catechin and phytosterols would not only ameliorate the antioxidant activity of catechins in lipophilic circumstance but also possibly integrate both of their beneficial activities. It has been unveiled that antioxidants with the amphiphilic structure exhibited intriguing antioxidant activities in typical lipid-based systems such as bulk oils and emulsions (Shahidi & Zhong, 2011). Integration of catechins and phytosterols would confer the amphiphilic structure to the molecules and afford amphiphilic antioxidants. Due to the nutritional values, it is essential to increase addition of polyunsaturated lipids in food products. However, their use in functional foods are often limited by the oxidative instability. Development of novel amphiphilic antioxidants will be highly desirable to protect polyunsaturated fatty acids from oxidation in bulk lipids or emulsions.

Thus, integration of (+)-catechin with the typical phytosterol, β-

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sitosterol, was investigated in this study. Connection of β-sitosterol and (+)-catechin was performed through a linkage of succinic diester chain. The antioxidant activities of the novel antioxidant to emulsions of polyunsaturated lipids were probed compared with (+)-catechin, β-sitosterol, ascorbic acid and typical lipophilic antioxidant TBHQ, employing fluorescence, β-carotene bleaching methods. The details were disclosed as follows.

2. Materials and methods

2.1. Chemicals

(+)-Catechin hydrate was purchased from Sigma-Aldrich. β-Sitosterol (> 75%), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N,N′*-Dicyclohexyl carbodiimide (DCC), ascorbic acid, linoleic acid, lauroyl chloride, 5-aminofluorescein, *N,N*-dimethylaminopyridine (DMAP) and 2,2′-azobis (2-amidinopropyl) dihydrochloride (AAPH) were purchased from Aladdin-Reagent (Shanghai, China). β-Carotene was purchased from Shanghai Macklin Biochemical (Shanghai, China). Tween-20 was obtained from Shanghai Zhanyun Chemistry (Shanghai China). MDA-kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other reagents were purchased from Sinopharm. All reagents were of analytical grade unless otherwise specified.

2.2. Preparation of (+)-catechin-β-sitosterol (CS)

β-Sitosterol (2074 mg, 5 mmol) and K_2CO_3 (1380 mg, 10 mmol) were dissolved into 35 mL *N,N*-dimethyl formamide (DMF). Then, succinic anhydride (1500 mg, 15 mmol) was added to the above solution. After stirred for 3.5 h at 50 °C, the reaction mixture was extracted by ethyl acetate, washed sequentially with 1 M aqueous HCl solution and saturated aqueous $NaHCO_3$ solution, dried over $MgSO_4$. Evaporation of the solvent afforded compound **1**.

To a solution of (+)-catechin (1450 mg, 5 mmol) in 40 mL *tert*-butyl methyl ether (t-BME) was added triethylamine (2896 μL, 22.5 mmol) dropwise, followed by the addition of propionic anhydride (5534 μL, 40 mmol). The reaction mixture was stirred for 15 h at room temperature. Then the crude product was extracted by ethyl acetate, washed with 1 M HCl and saturated aqueous $NaHCO_3$ solution, dried over $MgSO_4$. Purification by chromatography (silica gel, petroleum ether/ethyl acetate 3:1) afforded compound **2**.

The compound **2** (771 mg, 1.5 mmol) and compound **1** (849.3 mg, 1.65 mmol) in toluene (20 mL) was mixed with EDC (927 mg, 4.5 mmol) and DMAP (18 mg, 0.15 mmol) sequentially. The reaction mixture was stirred for 1 h at 40 °C. Then the product was extracted by EtOAc, washed with 1 M HCl and dried over $MgSO_4$. After evaporation of the solvent, compound **3** was purified by silica gel column chromatography (petroleum ether/ethyl acetate, 3:1).

The compound **3** (1011 mg, 1 mmol) was treated with a mixture of methanol (9 mL) and tetrahydrofuran (9 mL) along with addition of hydrazine hydrate (581 μL, 12 mmol). After stirred for 3 h at 30 °C, the reaction mixture was washed with 1 M HCl and dried over $MgSO_4$ to finally produce compound **4**, (+)-catechin-β-sitosterol (CS).

2.3. Structure determination of (+)-catechin-β-sitosterol (CS)

The chemical structure of CS was identified by NMR, MS, and IR spectroscopies. ^{1}H NMR and ^{13}C NMR spectra were recorded on a 500 MHz Bruker NMR spectrometer at room temperature in DMSO-d_6 with the solvent residual peak as internal reference (DMSO-d_6: ^{1}H = 2.50 ppm, ^{13}C = 39.52 ppm) (Gottlieb, Kotlyar, & Nudelman, 1997).

The UHPLC-MS analysis was performed on an Agilent 6460 Triple Quadrupole MS System (Agilent, Santa Clara, CA, USA). An Agilent ZORBAX Eclipse XDB-C18 (150 * 2.1 mm, 3.5 μm) column was applied. The column temperature was maintained at 40 °C and the sample injection volume was 5 μL. The mobile phase in UHPLC-MS determinations comprised formic acid aqueous solution (0.1%) (A) and methanol (B). The gradient elution profile started with A-B (10:90), after 10 min B was gradually increased to 95% within 15 min, then maintained for 20 min. The mobile phase was delivered at a flow rate of 0.4 mL/min and signals were monitored at 277 nm with DAD detection.

The IR spectroscopy analysis was performed on a FT-IR Bruker Tensor 27 spectroscopy (Bruker Optik Gmbh, Ettlingen, Germany) with a KBr disk containing 1% finely ground samples, and peaks are reported (cm^{-1}) with the following relative intensities: s (strong, 70–100%), m (medium, 40–70%), w (weak 20–40%).

2.4. Differential scanning calorimetry (DSC) analysis

The thermal behavior of samples was measured by DSC system which was accomplished by a model Q2000 calorimeter (TA Instrument, USA). Briefly, each sample (2–3 mg) was placed in a hermetic aluminum pan, heated from 25 °C to 200 °C at a rate of 10 °C/min. Nitrogen was employed as purge gas at a constant flow rate of 25 mL/min. A hermetic empty aluminum pan was considered as a reference. T_o (onset melting temperature), T_p (peak melting temperature), and ΔH (molar enthalpy) applied to describe characteristic temperatures of transitions were recorded by the instrument software according to each thermal curve.

2.5. Stability of (+)-catechin-β-sitosterol under acidic and basic conditions

The acidic disodium hydrogen phosphate-citric acid buffer (15 mM, pH 2.0) and basic phosphate buffered saline (PBS) buffer (15 mM, pH 7.5) were prepared. Then solutions of (+)-catechin-β-sitosterol (1 mM) were prepared by each buffer with addition of 5% Tween-20 to facilitate solubilization. The prepared solutions were stored at ambient temperature. The stability of (+)-catechin-β-sitosterol under these conditions was investigated by UHPLC analysis at 0, 6, 12, 24, 48 h after preparation. For UHPLC analysis, an aliquot (1 mL) of the corresponding solution was applied and the aliquot under basic condition was neutralized with addition of 0.2 mL of citric acid solution (0.1 M) before test.

The UHPLC analysis was carried out employing an Agilent ZORBAX Eclipse XDB-C18 (150 × 2.1 mm, 3.5 μm) column. The column temperature was maintained at 25 °C and the sample injection volume was 5 μL. The mobile phase in UHPLC determinations comprised formic acid aqueous solution (0.1%) (A) and methanol (B). The gradient elution profile started with A-B (10:90), after 10 min B was gradually increased to 95% within 15 min, then maintained for 20 min. The mobile phase was delivered at a flow rate of 0.4 mL/min and signals were monitored at 277 nm with DAD detection.

2.6. Synthesis of 5-dodecanoylaminofluorescein (DAF)

Triethylamine (239.2 μL, 1.73 mmol) was directly added to a solution of 5-aminofluorescein (200.0 mg, 0.58 mmol) in *N,N*-dimethyl formamide (10 mL), followed by addition of lauroyl chloride (266.6 μL, 1.16 mmol). The mixture was stirred for 2.5 h at the room temperature. Then, the compound was extracted with ethyl acetate, washed with 1 M HCl and dried over $MgSO_4$. Finally, DAF was concentrated in vacuo after filtration. (Chaiyasit, Mcclements, Weiss, & Decker, 2008)

2.7. Measurement of partition coefficient (log P) in octanol/PBS

Solutions (1.5 mM) of each compound in 1-octanol were warmed at 60 °C for 1 h and the absorbance at 254 nm was read by UV spectrum (A_0). An equal volume of phosphate-buffered saline (0.1 M, pH 6.0) was added to the solution prepared above. The resultant mixture was vortexed for 1 min and centrifuged at 1000 rpm for 10 min. Absorbance