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Lipophilization of EGCG and effects on antioxidant activities

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2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, PubChem CID: 9570474)
6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Pubchem CID: 40634).

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ABSTRACT

A green, fast, and efficient method for synthesizing lipophilic epigallocatechin gallate (EGCG) derivatives was set up for the first time. EGCG was lipophilized by esterification in order to promote its application in lipid products and to possibly enhance its bioactivity. A high conversion of EGCG was achieved. Three monoesters of the EGCG derivatives were confirmed by high performance liquid chromatography-mass spectrometry, and the predominant one was identified as 4′-*O*-palmitoyl EGCG by nuclear magnetic resonance. The EGCG derivatives exhibited good radical scavenging capacities. In lard the solubility of EGCG derivatives was enhanced 470 times compared to EGCG, and they exhibited excellent antioxidative activity in the oil. These results indicate that the palmitoylated EGCG derivatives may be used as potent antioxidants in lipophilic medium, such as edible oils and fatty foods. In addition, this method can be applied to commercial application, producing antioxidants to substitute for synthetic ones like *tert*-butylhydroquinone.

1. Introduction

(−)-Epigallocatechin gallate (EGCG), the most abundant catechin (Wang, Helliwell, & You, 2000) in green tea leaves, has excellent biologic activities, including anticancer, antioxidant, antiviral, and anti-inflammatory activities (Friedman, 2007; Nichols & Katiyar, 2010; Shahidi & Ambigaipalan, 2015; Wang & Bachrach, 2002). Moreover, epidemiological studies suggest that intake of EGCG can lower the risk of cardiovascular disease, neurodegenerative diseases, diabetes and obesity (Deka & Vita, 2011; González-Castejón & Rodriguez-Casado, 2011; Kamiyama et al., 2010; Mandel, Amit, Weinreb, & Youdim, 2011). However, EGCG is unstable in neutral or alkaline medium (Lam et al., 2004), and the eight hydroxyls of EGCG make it hard to dissolve in oil, which attenuates its effectiveness in protection of lipid oxidation, as well as absorption by human body (Tanaka, Kusano, & Kouno, 1998).

Lipid oxidation is a major cause of food quality deterioration and cause of initiation and propagation of health issues due to oxidative stress (Shahidi & Zhong, 2005). However, traditional synthetic antioxidants like *tert*-butylhydroquinone (TBHQ) were found to have adverse effects on health (Kashanian & Ezzati, 2009). Many countries have restricted or prohibited the use of synthetic antioxidants due to the potential toxicity and carcinogenicity. Thus, natural antioxidants alternatives have attracted recent attention.

Esterification has been selected as the most efficient means to modify EGCG molecule, thus various efforts have been made on esterification of EGCG to increase its lipid solubility, as well as chemical/metabolic stability and bioavailability. Prior work has showed that EGCG fatty acyl esters are more stable than EGCG to oxidation (Zhong & Shahidi, 2011). Besides, EGCG ester derivatives can be decomposed into EGCG, but exerts significantly enhanced anticancer effects than EGCG, suggesting their potential use as prodrugs of EGCG (Lam et al., 2004; Landis-Piwowar et al., 2007).

The modification methods of EGCG molecule include enzymatic catalysis and chemical catalysis. For enzymatic catalysis, it is known that lipases have good regioselectivity. However, when the substrates have multiple reaction sites, the products may be complicated, since mono-, di- and tri-esters are produced (Chebil et al., 2007). Moreover, enzymatic catalysis has some disadvantages, such as the lipases are expensive and time-consuming, and using toxic solvents sometimes (Zhu et al., 2013). As for chemical catalysis, it is fast, inexpensive, and having higher yields, but often uses pyridine or triethylamine as the organic base to react with the hydrogen chloride released in the

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reaction (Mori et al., 2008; Zhong & Shahidi, 2011). However, the organic bases above are usually toxic and hardly completely removed in the process of purification. Based on these studies, we determined to search for a suitable chemical catalyst/base for esterification of EGCG.

So far, the catalysts/base of sodium acetate, the solubility of the palmitoylated EGCG derivatives in edible oils, and their potential as food antioxidants have not been reported. The aim of this work is to introduce an efficient chemical catalysis method on esterification of EGCG to EGCG palmitate derivatives. The radical scavenging capacity and antioxidative activity of the EGCG derivatives were evaluated and compared to common used antioxidants.

2. Materials and methods

2.1. Materials

EGCG (95%) was supplied by Pulimeidi Inc. (Hangzhou, China). Palmitoyl chloride, *tert*-butylhydroquinone (TBHQ) and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were acquired from Aladdin (Shanghai, China). Sodium acetate was purchased from Sinopharm Group Chemical Reagent Co. (Shanghai, China). Edible lard oil without additives was purchased from the Jin'en Inc. (Wenzhou, China). Acetonitrile of HPLC grade was purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the other solvents and chemicals used were obtained from Sinopharm Group Chemical Reagent Co. (Shanghai, China) and were of analytical grade.

2.2. Preparation of EGCG derivatives

In a typical one-step synthesis protocol, 10 mmol EGCG (4.83 g) was added to 100 mL of acetone and heated in a water bath at 40 °C. After complete dissolution, 2.46 g of sodium acetate was added to the solution. Subsequently, two molar ratio of palmitoyl chloride was dropwise added to the solution, with stirring. The mixture was carried out in a stoppered flask, in a water bath condition, and under no nitrogen blanket. After reacting for 6 h, the reaction mixture was filtered and washed with 100 mL deionized water. Then 100 mL ethyl acetate was added to extract the mixture. And the organic phase was washed 3 times with deionized water. After then, the upper organic phase was dried with anhydrous sodium sulfate and concentrated under reduced pressure. Faint yellow powdery product was obtained.

2.3. HPLC-MS analytical procedure

The composition of the EGCG derivatives was determined by reversed phase HPLC-MS, using an Agilent 1290 HPLC unit (Agilent Technologies, Palo Alto, USA) with a UV detector (1290 VWD). Separation was achieved on a Cosmosil ODS C18 column (4.6 mm × 250 mm, 5 μm; Nacilai Tesque Inc., Japan). Eluent A and eluent B were acetonitrile/water = 80:20 (v/v), and acetonitrile/water = 10:90 (v/v), each containing 0.2% formic acid (v/v). A gradient programme was as follows: 0–20 min, linear gradient 0–10% A; 21–60 min, 88% A isocratic. The flow rate was 1.0 mL/min and fractions were detected at 275 nm. LC flow was further analyzed online by a 6460 Triple Quad MS detector system (Agilent Technologies, Palo Alto, USA) with electrospray ionization (ESI) interface at negative mode. The effluent was entered into an electrospray source (desolvation temperature, 325 °C; capillary voltage, 3.5 kV; nebulizer, 45 psi). Argon was used as collision gas (collision energy 16 eV), and nitrogen was the desolvation gas (dry gas flow, 5 L/min).

2.4. Reaction process monitoring

The conversion constant of EGCG was calculated according to the normalized peak area in each HPLC chromatogram. The HPLC determination was carried out on a Wufeng LC-100 HPLC (Shanghai, China) coupled with Wufeng LC-100 UV detector (Suzhou, China), equipped with a Cosmosil ODS C18 column (4.6 mm × 250 mm, 5 μm; Nacilai Tesque Inc., Japan). The UV detector was set at the wavelength of 275 nm. The column temperature was 30 °C. Eluent A and eluent B was acetonitrile/water = 80:20 (v/v), and acetonitrile/water = 10:90 (v/v), each containing 0.2% formic acid (v/v). A gradient programme was as follows: 0–20 min, linear gradient 0–10% A; 21–60 min, 88% A isocratic. The flow rate was 1.0 mL/min.

After reacting for 2, 4 and 6 h, 5 mL of the reaction mixture was respectively washed with deionized water, extracted with ethyl acetate, washed three times with deionized water and then concentrated under reduced pressure. 20 μl of the above concentrated sample was subjected to HPLC analysis. Chromatography provided the normalized area of each peak, from which a relative content of the peak can be obtained, thus the EGCG to EGCG derivatives conversion constant can be obtained.

2.5. Purification and identification of EGCG derivatives

Flash column chromatography was used to separate individual EGCG derivatives. EGCG derivatives were eluted on a silica column with a mixture of toluene/ethyl acetate/acetic acid (80:20:1 v/v/v). The fractions were analyzed by TLC (toluene/ethyl acetate/acetic acid = 50:50:1, v/v/v), and one predominant fraction was collected, washed three times with deionized water. Solvents were removed using a rotary evaporator. The predominant fraction of the EGCG derivatives (referred to as compound 1) was analyzed for its specific structure.

2.6. NMR analyses

The ^{1}H (400.13 MHz) and ^{13}C (100.61 MHz) and heteronuclear multiple-bond correlation (HMBC) spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer (Bruker Biospin Co., Billerica, USA). The samples were dissolved in DMSO-d_6 containing TMS as an internal standard. The structure elucidation was accomplished by comparing the chemical shifts of EGCG derivatives with the parent EGCG molecule. HMBC analysis was carried out to identify the location of fatty acid incorporated in the EGCG molecule.

2.7. Determination of lipophilicity of EGCG derivatives

Due to the fact that the EGCG derivatives were a mixture, the lipophilicity of 4′-*O*-palmitoyl EGCG was calculated. The lipophilicity of the EGCG and 4′-*O*-palmitoyl EGCG was computationally obtained by ALOGPS 2.1 (Tetko & Bruneau, 2004). The structures of EGCG derivatives in simplified molecular input entry (SMILE) system were obtained by Chem Draw Std 14.0.

2.8. Determination the oil solubility of EGGC and its derivatives

The oil solubility of EGCG derivatives and EGCG was measured by direct observation. The method was described as follows: 1 g sample was put into a test tube while stirred and heated to 30 and 90 °C, respectively. Then edible lard was dropwise added until all samples were dissolved. Then the oil amount added was noted down. The oil solubility was expressed as g/100 g at 30 and 90 °C, respectively.

2.9. ABTS radical cation scavenging assay

ABTS radical cation ($ABTS^{\cdot+}$) scavenging test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. Furthermore, the ABTS radical is soluble in water and organic solvents, thus enabling it applicable for both lipophilic and hydrophilic compounds (Arnao, Cano, & Acosta, 2001;