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Biotransformation of phlorizin by human intestinal flora and inhibition of biotransformation products on tyrosinase activity

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ABSTRACT

Phlorizin (**1**) was anaerobically incubated with human intestinal bacteria and five biotransformation products (**2**–**6**) were obtained. Their structures were elucidated as phloretin (**2**), phloroglucinol (**3**), phloretic acid (**4**), 2,3,4-trihydroxybenzenepropanoic acid (**5**), and phloretic acid methyl ester (**6**) on the basis of their spectroscopic data. Using high-performance liquid chromatography equipped with a diode array detector, chromatographic separation of **1**–**4** was performed on an analytical C_{18} column. The time course of the biotransformation was studied to probe into the biotransformation mechanism of **1** by human intestinal flora. The abilities of isolated strains to transform **1** were also investigated in order to find an optimal transformation strain. In addition, the inhibitory activity of parent compound **1** and its three main biotransformation products **2**–**4** on mushroom tyrosinase was estimated. The result showed that **2** has better inhibitory effect on tyrosinase activity than **1**.

1. Introduction

Phenolic compound phlorizin, a natural product and dietary constituent found in a number of plants, is a prominent member of the chemical class of dihydrochalcones. It consists of a glucosyl moiety and a C_6–C_3–C_6 skeleton structure (Fig. 1). As the principal phenolic-glucoside in apple trees, phlorizin is distributed in root bark, shoots, leaves (Hutchinson, Taper, & Towers, 1959), and seeds (Lu & Foo, 1998). It is also present in processed apple products such as apple juice and cider (Peng, Liu, Peng, & Ye, 2005), and by-products such as apple pomace (Lu & Foo, 2000; Diñeiro García, Suárez Valles, & Picinelli Lobo, 2009; Suárez et al., 2010). The most important pharmacological action of phlorizin is to block glucose absorption or reabsorption through competitive inhibition of the sodium–glucose symporters located in the mucosa of the small intestine and proximal renal tubule and therefore to produce glycosuria (Panayotova-Heiermann, Loo, & Wright, 1995). Due to its potential application as a drug and tool for physiology research, phlorizin has gained widespread attention from the research community recently. More than 600 research articles in relation to various pharmacological effects of phlorizin and its derivatives have been published since the new millennium. These studies focused on its effects on diabetes, obesity, stress hyperglycaemia, antioxidative activity, and membrane permeability. Owning to all these significant effects, phlorizin could be potentially applied in fields of foods, beverages, food additives, pharmaceuticals as well as cosmetics (Sukhorukov et al., 2001; Valenta, Cladera, O'Shea, & Hadgraft, 2001; Rezk, Haenen, van der Vijgh, & Bast, 2002; Ehrenkranz, Lewis, Kahn, & Roth, 2005). Besides, scientists have found that phlorizin also has inhibitory effects on the activity of mushroom tyrosinase (Wang et al., 2007). Tyrosinase, widely distributed in microorganisms, animals and plants, is the precipitating factor for both browning in plants and melanization in animals. The inhibitors of this enzyme could be used for the prevention and treatment of some dermatological disorders like melanoma (Minekoa et al., 1992) and for whitening and depigmentation after sunburn in cosmetics industry (Maeda & Fukuda, 1991). Phlorizin was found to have a high inhibitory effect on both phases – monohydroxy and *o*-dihydroxyphenolase activity of tyrosinase (de Faria, Moure, Lopes, Krieger, & Mitchell, 2007; Wang et al., 2007). According to the published paper (Wang et al., 2007), the concentration of phlorizin leading to 50% diphenolase activity inhibition (IC_{50}) was estimated to be 110 μM.

In contrast, knowledge about the intracorporal course of phlorizin is limited, especially in the metabolism course of human intestinal flora. It's well known that intestinal flora plays a key role in the metabolism of foods and drugs administrated orally. Before being absorbed through the intestinal barrier, compounds and/or drugs may be transformed by intestinal flora leading to the occurrence of new more active or toxic compounds. This course is vital for whether or not potential drugs like phlorizin can be developed into clinical application. Also it is a good way to find new lead

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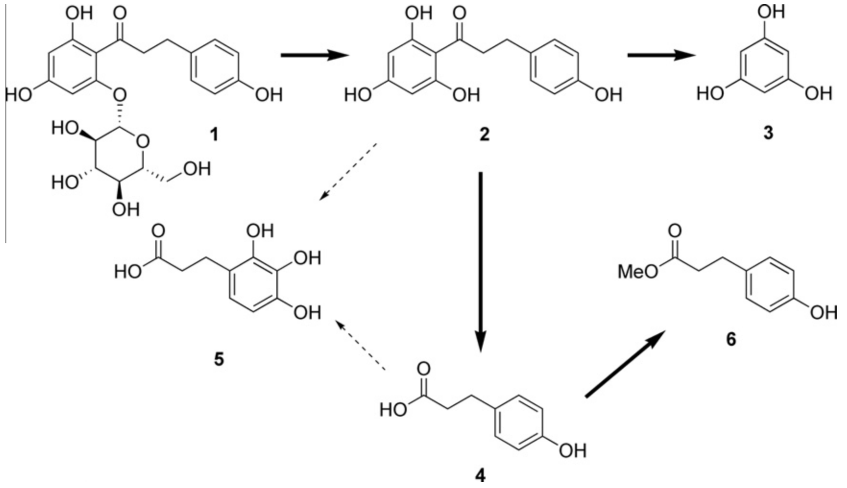

Fig. 1. Chemical structures of phlorizin (**1**) and biotransformation products (**2**–**6**), and possible biotransformation pathways for their production.

compounds based on the intracorporal course of drugs. Therefore, in this study, we focused on the study of biotransformation of phlorizin by the incubated human intestinal flora, and carried out a series of *in vitro* experiments to investigate the intracorporal metabolism course of phlorizin. Besides, the inhibitory activity of both phlorizin and its biotransformation products on mushroom tyrosinase have been studied in order to evaluate the effect of the biotransformation.

2. Materials and methods

2.1. Sample material, standards and reagents

Sodium thioglycolate, L-cysteine hydrochloride, and glucose were purchased from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China). Tryptone, beef extract, beef liver extract powder, digestibility serum powder, soya peptone, yeast extract, and proteose peptone were purchased from Beijing Shuangxuan Microorganism Medium Product Factory (Beijing, China). Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma (St. Louis, MO, USA). L-3,4-Dihydroxyphenylalanine (L-DOPA) were purchased from National Institutes for Food and Drug Control (Beijing, China). Dimethylsulphoxide (DMSO) was the product of Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade methanol (MeOH) and acetonitrile (MeCN) were purchased from Tianjin Xihua Chemicals Co. (Tianjin, China). All other analytical grade reagents were from Beijing Chemical Reagent Company (Beijing, China). Water (H_2O) was milli-Q grade.

Crude phlorizin with 80% of purity was kindly provided by Tianjin Jianfeng Natural Product R & D Co. Ltd. (Tianjing, China).

2.2. Methods

2.2.1. General procedures

The UV spectra were measured on a Varian Cary-300 ultraviolet-visible photometer. 1D and 2D NMR spectra were recorded on a Bruker AVANCE III 400 (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) using DMSO-d_6 as solvent, with TMS as internal standard. Mass spectra were recorded on an ABI MDS SCIEX API/QSTAR mass spectrometer for ESIMS and a Finnigan TRACE 2000 mass spectrometer for EIMS. A Thermo Scientific 1029 Forma Anaerobic System was used to create anaerobic conditions and Oxoid BR0055 anaerobic indicator was used. A semi-preparative reversed phase HPLC system including a LC P600 pump, a UV600 UV–Vis detector and Labtech Chrom software (LabTech Co., Beijing, China), equipped with a column Phenomenex Luna 10 C_{18} (2) (21.2 × 250 mm, 10 μm, Phenomenex Inc., Torrance, CA, USA) was used to isolate compounds from the extracts of bacterial mixture. Analytical HPLC was performed on a Varian chromatographic system including a Varian ProStar 230 HPLC pump, a Varian ProStar 330 PDA detector, and a Rheodyne injector with a 20 μl sample loop. The results were recorded using a Varian Star Chromatography Workstation and computer software (version 4.5). Open column chromatography (CC) separation was carried out using silica gel (200–300 mesh; Qingdao Marine Chemical Co., Qingdao, China), Sephadex LH–20 (Pharmacia, Fine Chemicals, Inc., Piscataway, NJ, USA) as stationary phase. TLC was conducted on silica gel GF_{254} plates (Merck).

2.2.2. Purification of phlorizin

Crude phlorizin (150 g) was subjected to silica gel CC, eluted with $CHCl_3$–MeOH (8:1, v/v) to afford three fractions. Fraction 2 was recrystallized with MeOH to obtain highly purified phlorizin (128.18 g). Its purity was over 99% determined by HPLC method and the chemical structure was determined by IR, MS, and NMR spectra. Phlorizin (**1**) was isolated as a white amorphous powder (MeOH). $C_{21}H_{24}O_{10}$, IR (KBr) cm^{-1} 3376, 2934, 1628, 1516, 1462, 1374, 1197, 835, 644. positive ESIMS *m/z* 437 $[M + H]^+$, 275 $[\text{aglycone} + H]^+$; negative ESIMS *m/z* 435 $[M-H]^-$, 273 $[\text{aglycone}-H]^-$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.78 (2H, t, $J = 7.4$ Hz, H-β × 2), 3.21 (1H, m, H-2″), 3.28 (1H, m, H-4″), 3.32 (1H, m, H-3″), 3.35 (1H, m, H-5″), 3.38 (2H, m, H-α × 2), 3.50 (1H, dd, $J = 5.6$, 10.2 Hz, H_a-6″), 3.70 (1H, dd, $J = 3.6$, 10.2 Hz, H_b-6″), 4.58 (1H, dd, $J = 5.6$, 6.0 Hz, OH-6″), 4.93 (1H, d, $J = 7.2$ Hz, H-1″), 5.03 (1H, d, $J = 5.2$ Hz, OH-2″), 5.13 (1H, d, $J = 4.4$ Hz, OH-3″), 5.28 (1H, d, $J = 5.2$ Hz, OH-4″), 5.92 (1H, d, $J = 2.2$ Hz,, H-3′), 6.12 (1H, d, $J = 2.2$ Hz, H-5′), 6.63 (2H, d, $J = 8.4$ Hz, H-2, H-6), 7.02 (2H, d, $J = 8.4$ Hz, H-3, H-5), 9.08 (1H, s, OH-4), 10.56 (1H, s, OH-4′), 13.49 (1H, s, OH-6′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 205.2 (C=O), 45.4 (C-α), 29.4 (C-β), 132.0 (C-1), 129.6 (C-2), 115.4 (C-3), 155.7 (C-4), 115.5 (C-5), 129.6 (C-6), 105.6 (C-1′), 165.8 (C-2′), 97.1 (C-3′), 164.9 (C-4′), 94.6 (C-5′), 161.3 (C-6′), 101.3 (glc-C-1), 73.7 (glc-C-2), 77.3 (glc-C-3), 69.9 (glc-C-4), 77.1 (glc-C-5), 60.8 (glc-C-6).

2.2.3. Biotransformation of **1** and identification of the products

The formula of the general anaerobic medium (GAM) broth, procedure of the preparation of GAM, and human intestinal bacterial mixture were undertaken according to the previously paper of our laboratory (Zhang & Yang, 2009). An activated bacterial flora (10 ml) was added to GAM solution (900 ml) containing **1**