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In vitro biotransformation of red ginseng extract by human intestinal microflora: Metabolites identification and metabolic profile elucidation using LC–Q-TOF/MS

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ABSTRACT

Ginseng is an important and widely used herbal medicine in Asia and has gained popularity in the western countries. Ginseng products are usually administered orally, after which their complicated components are brought into contact with intestinal microflora in the alimentary tract and metabolized. The metabolic investigation of ginseng in intestinal tract is necessary for elucidating its pharmacological activities. However, most of the reports about the metabolism of ginseng with intestinal microflora are focused on single ginseng saponin with the whole action of ginseng extract ignored. In the present paper, *in vitro* biotransformation of red ginseng extract by human intestinal microflora was conducted, and a rapid liquid chromatography with time-of-flight mass spectrometry (LC–Q-TOF/MS) method was used for rapid identification of the metabolites and metabolic profile of ginseng saponins. A total of 37 ginseng saponins in red ginseng extract were characterized, 17 of which were assessed to be metabolized by human intestinal microflora. Also, 30 metabolites, mostly deglycosylated, were detected and identified in the biotransformed red ginseng extract, including 4 original ingredients of red ginseng, 6 ginsenoside lactate esters, and 2 glycosylated metabolites. The metabolic profile of ginseng saponins biotransformed by human intestinal microflora was elucidated based on the metabolite information. The results indicated that deglycosylation was the major metabolic pathway of saponins in red ginseng. The esterification and glycosylation reaction also occurred during the biotransformation. Our study indicated that there was some differences in the biotransformation of single ginseng saponin and red ginseng extract. It must be noted that the ginsenoside lactate esters were firstly found in the metabolites of ginsenosides.

1. Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, is one of the most commonly used herbal medicines in Asia. The major components of ginseng are ginseng saponins, which have been reported to show various biological activities including anti-inflammatory activity [1] and anti-tumor effects [2,3]. In Asia, two types of ginseng are widely used including white ginseng and red ginseng. Traditionally, white ginseng is produced from fresh ginseng by dehydration in sunlight, and red ginseng is manufactured by steaming fresh ginseng at 95–100 °C for 2–3 h followed by drying. Red ginseng was reported to have better bioactivity than white ginseng, which may result from the chemical transformation of ginseng saponins during the heating treatment [4,5]. Because of its excellent efficacy, red ginseng was widely used in some traditional Chinese medicine (TCM) formulas, such as Shenmai injection [6], Shengmai injection [7], and Shenfu injection [8], for the treatment of cardiovascular diseases or cancer.

It is well known that intestinal microflora play an important role in the metabolism of compounds administered orally or excreted into bile. In TCMs, most crude drugs are orally administered as decoctions. Therefore, their components are inevitably brought into contact with intestinal microflora in the alimentary tract and are metabolized by intestinal microflora before absorption from the intestinal tract into the blood [9,10]. Thus, intestinal microflora has extensively been used for the *in vitro* metabolic study of natural products, such as saponins, flavonoids, quinones, terpenoids, and alkaloids [10–15]. For ginseng, its products are often taken orally as functional food in thermally prepared forms, such as white

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ginseng, red ginseng, and ginseng extract. Although some of ginseng saponins are transformed in the stomach by gastric juice *via* deglycosylation, dehydration, hydration, and oxygenation reaction, most of the orally administered ginseng saponins inevitably come into contact with intestinal microflora and enzymes in the intestinal tract, and may be transformed before they are absorbed from gastrointestinal tract [16–18]. The pharmacological effects of ginseng saponins have been partly explained by the biotransformation of ginseng saponins with human intestinal microflora. For example, compound K transformed from ginsenoside Rb_1, Rb_2 and Rc was previously reported to have better anti-inflammatory and anti-tumor effect [1,19–21]. The number and species of intestinal microflora are different from individuals, depending on the conditions of the host, including diet, health, and even stress [19]. It has been reported that the metabolism of ginsenosides by intestinal microflora is variable between individuals, and probably be dependent on the composition of intestinal microflora [22]. In the present study, twenty healthy volunteers were selected to avoid the intestinal microflora diversity between individuals.

The characterization of the metabolites and metabolic profile of ginseng saponins in intestinal tract is important for explanation of the pharmacological actions of red ginseng extract. Degradation and metabolism of ginseng saponins have been conducted using gastric acidic conditions, enzymes or intestinal microflora [10,18]. However, most of these reports only dealt with a single ginseng saponin with the whole action of ginseng extract ignored. Oligosaccharides are potential source of prebiotics. Therefore, the oligosaccharides from the ginseng may affect the composition of intestinal microflora, thus influence the metabolism of ginseng saponins [23,24], and the metabolites of red ginseng extract probably be different from single ginseng saponin. Few reports have investigated the comprehensive metabolites and metabolic profile of red ginseng extract, especially for the identification of minor metabolites, probably due to the chemical complexity of metabolites, the lack of reference standards, or the inherent limitation of analytical methods. To further clarify the metabolic profile of red ginseng saponins biotransformed by human intestinal microflora, a rapid liquid chromatography with quadrupole time-of-flight mass spectrometry (LC–Q-TOF/MS) method, which provides accurate masses of ions and valuable structural information [11,15], could be used to characterize the ginseng saponins and its biotransformed metabolites.

In this paper, an *in vitro* biotransformation study of red ginseng extract by human intestinal microflora was conducted, and a highly sensitive and selective LC–Q-TOF/MS method was used for rapid identification of the metabolites and metabolic profile of ginseng saponins. The present study may provide new insights for the study of metabolism and active metabolites of red ginseng.

2. Materials and methods

2.1. Chemicals and reagents

General anaerobic medium broth (GAM broth), vitamin K_1 and hematin chloride were purchased from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China). HPLC grade acetonitrile (ACN) and formic acid were obtained from Merck (Darmstadt, Germany). Deionized water (18 MΩ/cm) was supplied with a Millipore Milli-Q water system (Milford, MA, USA). Other reagents were of analytical purity.

Reference standards of ginsenoside Rb_1, Rb_2, Rb_3, Rc, Rd, Re, Rg_1, F_1, F_2, 20*S*-Rg_2, 20*R*-Rg_2, 20*S*-Rg_3, 20*R*-Rg_3, 20*S*-Rh_1, 20*R*-Rh_1, 20*S*-Rh_2, 20*R*-Rh_2, compound K, protopanaxatriol and protopanoxadiol were purchased from Jilin University (Changchun, China); other reference standards, including ginsenoside Rk_3, Rh_4, Rk_1 and Rg_5, were provided by Professor Lian-Wen Qi of China Pharmaceutical University. Their structures were shown in Fig. 1 , and the purity of each reference standard was determined to be more than 95% by normalization of the peak areas detected by HPLC-DAD-TOF/MS and the reports of ^{13}C-NMR analysis.

2.2. Red ginseng extract preparation

Red ginseng originated in Jilin province of China was purchased from Anhui Jiren Pharmaceutical Co., Ltd. (Anhui, China). The voucher samples were authenticated by Professor Bo-Yang Yu and deposited at the Department of Complex Prescription of TCM at China Pharmaceutical University (Nanjing, China).

The red ginseng samples were pulverized into powder. Ten grams of the powder were extracted in an ultrasonic bath with 100 ml 80% ethanol at room temperature for 1 h. The operation was repeated twice, and the combined extracts were evaporated under vacuum and lyophilized. The sample was stored at 4 °C and re-diluted in 10 ml sterile water prior to use. Then the sterile water solution of red ginseng sample was filter through a 0.22 μm-pore-sized filter (Millipore, type GV), the filtrate were collected in a sterile tube and used for *in vitro* biotransformation vessels.

2.3. Human fecal sample preparation

Fresh human feces samples were collected from twenty healthy Chinese volunteers (20–30 years, 11 males and 9 females) from the Department of Complex Prescription of TCM, China Pharmaceutical University (Nanjing, China). All volunteers were in good health and had not been given antibiotics for at least 6 months before the study. Samples were collected and mixed, on site, on the day of the experiment and were used immediately. Fecal slurries were prepared by mixing fresh feces samples with autoclaved PBS (0.1 M, pH 7.2) to yield 10% (w/v) suspensions. The fecal suspensions were filtered with two layers gauze. The filtered suspensions were then used to inoculate the *in vitro* biotransformation vessels.

2.4. In vitro biotransformation of red ginseng extract by human intestinal microflora

A 30 g GAM broth was dissolved in 1000 ml water (70 °C), filtrated while hot, after treated with anti-bacteria process with high pressure (0.15 MPa) and temperature (121 °C) for 20 min, cooled to 45 °C. The GAM broth solution was then transferred to an anaerobic chamber (37 °C, anaerobic condition), and 1 mg vitamin K_1 and 6 mg hematin chloride were dissolved in the solution. Then biotransformation vessels (50 ml volume; one vessel per experiment group) were sterilized and filled with 30 ml of GAM broth solution.

Vessels were inoculated with 3 ml of fecal suspensions (1:10, w/v) and then 1 ml of sterile red ginseng extract was added. *In vitro* biotransformation was run under anaerobic conditions for a period of 48 h. Two different control experiments were conducted: (1) incubations of the intestinal microflora in medium, but lacking the red ginseng extract, to monitor metabolites arising from basal metabolism; (2) incubations of the red ginseng extract in medium but without intestinal microflora, to monitor changes due to the non-microbial chemical transformation of precursor compounds of the substrate.

The biotransformation mixtures were then prepared according to the method described in the literature [15] with some modifications. Briefly, the biotransformation mixtures were extracted by 50 ml ethyl acetate for three times. The remaining residues were re-extracted three times with 50 ml water-saturated *n*-butanol. The combined *n*-butanol layers were washed with water three times. Then the ethyl acetate and *n*-butanol layers were mixed homogeneously and concentrated under vacuum, and then diluted to the