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Identification of the major metabolites of hyperoside produced by the human intestinal bacteria using the ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry

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ABSTRACT

Ethnopharmacological relevance: Traditional Chinese medicine (TCM), used in clinical practice for thousands of years, has been taken as a new way to tackle chronic diseases. In Chinese medicine, many ingredients which are known to have biological effects just pass through the gut, they do not get into the bloodstream. Study on interactions of these active ingredients with the intestinal bacteria is very helpful to unravel how TCM works.

Aim of the study: Hyperoside is a major active constituent in many medicinal plants which are traditionally used in Chinese medicines for their neuroprotective, anti-inflammatory and anti-oxidative effects. It would be metabolized by intestinal bacteria after oral administration which leads to the variation of ethnopharmacological profile of these traditional medicines. Thus, it is necessary to investigate the metabolic routes and metabolites of hyperoside produced by human intestinal bacteria.

Materials and methods: Ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/QTOF-MS) technique combined with Metabolynx™ software was used for analysis of the metabolic profile of hyperoside by the isolated human intestinal bacteria from the human feces.

Results: Parent compound and 6 metabolites (M1-M7) were detected in the isolated bacterial samples compared with blank samples. Quercetin, 3, 4-dihydroxyphenylacetic acid and 3, 4-dihydroxyphenylbenzoic acid (M2-M4) were only found in the sample of *Bacteroides sp.* 45. Dehydroxylation of hyperoside and the conjugates: acetylation and hydroxylation of hyperoside (M5–M7) were identified in the majority of isolated intestinal bacterial samples.

Conclusions: Only *Bacteroides sp.* 45 could metabolize hyperoside to form its aglycone quercetin, thereafter, quercetin was further metabolized to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid. Furthermore, the majority of bacteria could metabolize hyperoside to dehydroxylation of hyperoside and the conjugates: hydroxylation and acetylation of hyperoside. In this study, the metabolic routes and metabolites of hyperoside produced by the different intestinal bacteria were investigated for the first time. Furthermore, the production of metabolites of hyperoside might influence the effects of traditional medicines. Thus, the study on the metabolism of hyperoside by human intestinal bacteria is of great importance to an understanding of the effects of traditional medicines.

1. Introduction

Hyperoside is present in many medicinal plants such as *Hypericum perforatum* (Sosa et al., 2007), *Artemisia capillaris* (Park et al., 2012) and *Flos lonicerae* (Qian et al., 2007). These plants are used in classical traditional medicines such as *Hypericum perforatum* extracts, Yinchenhao decoction and yinqiaosan powder which have been used for centuries and widely adopted clinically to treat depressant (Butterweck et al., 2000), liver cirrhosis (Liu et al., 2008) and influenza diseases (Wang et al., 2010), respectively. Although it is well known that the therapeutic effects of traditional medicines are based on the synergistic actions of muti-compounds (Liu et al., 2012), hyperoside is also a key active compound to participate in the medicinal effects (Butterweck et al., 2000; Shi et al., 2001). Many pharmacologic actions of hyperoside might well contribute to the clinical effects of traditional medicines. There have been reported that hyperoside possessed anti-oxidant activity by scavenging free radicals (Zou et al., 2004), anti-inflammatory action through suppressed production of tumor necrosis factor, interleukin-6 and nitric oxide (Sosa et al., 2007; Kim et al., 2011), anti-depressant effect mediated by the dopaminergic system (Haas et al., 2011), anti-bacterial and anti-viral activities (Wu et al., 2007; Chen et al.,

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2012). In addition, hyperoside had hepatoprotective effect through the enhancement of anti-oxidative and anti-inflammatory systems (Choi et al., 2011).

Furthermore, most traditional medicines are oral administrated, their components are therefore inevitably brought to contact with intestinal bacteria in the intestinal tract, and are metabolized by intestinal bacteria before absorption from the intestinal tract into the blood. At least some of the ingredients in traditional medicines exert their biological effects through interactions with intestinal bacteria (Park et al., 2006). As hyperoside is an ubiquitous active flavonoid that is present in numerous medicinal plants, the metabolism of hyperoside produced by intestinal bacteria is of great importance to the biological effects of traditional medicines.

A part of the quercetin glycosides such as rutin (quercetin-3-*O*-rutinose) and isoquercitrin (quercetin-3-*O*-glucoside) were expected to reach the intestinal tract, where they were metabolized to quercetin and further metabolites phloroglucinol, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, and 3,4-dihydroxybenzaldehyde by the intestinal bacteria (Jaganath et al., 2009; Justesen and Arrigoni, 2011). However, the routes and metabolites of hyperoside produced by human intestinal bacteria are still not clear. Hyperoside converted by human intestinal bacteria is essential for the absorption, bioavailability, and bio-activities of this compound. There were reports about the metabolites and pharmacokinetic study of hyperoside in rat after intravenous administration (Liu et al., 2010; Guo et al., 2011). But Chang et al. (2005) found that hyperoside could not be detected in plasma of rats either as the unchanged form or as its aglycone or conjugated aglycone after administration of hyperoside at 6.0 mg/kg p.o. It might be the weak metabolic activity of intestinal bacteria to hyperoside. Moreover, Knaup et al. (2007) studied the influence of different sugar moieties of quercetin glycosides on the human ileal microflora hydrolysis of phenol glycosides, the 3-*O*-beta-D-glucopyranoside showed with 0.22 micromol/h hydrolysis rate, however the 3-*O*-beta-D-galactopyranoside showed with 0.04 micromol/h hydrolysis rate. From these results, we concluded that hyperoside could be metabolized by intestinal bacteria, in spite of the low metabolism rate. Therefore, our research group has carried out investigations on the in vitro metabolism of hyperoside by isolating different pure bacteria from human feces.

In this study, Ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/QTOF-MS) was used to analyze the metabolites of hyperoside by the different human intestinal bacteria. Quadrupole time-of-flight tandem MS plays a crucial role in the study of drug metabolism because it provides not only accurate masses of ions, but also gives valuable structural information from the MS/MS spectra (Shen et al., 2010; Han et al., 2011). By the results, we can know the probable metabolic routes and metabolites of hyperoside produced by the human intestinal bacteria.

2. Experimental

2.1. Materials

Hyperoside was purchased from Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China). The 99.34% purity of hyperoside was validated by this company in terms of NMR, HPLC, etc. HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, USA); formic acid was obtained from Merck KGaA (Darmstadt, Germany); ultra-pure water was freshly prepared with an EPED super purification system (Nanjing, China). Other reagents were of analytical grade. The General anaerobic medium (GAM) used in this study were as follows: 10.0 g tryptone, 3.0 g soya peptone, 10.0 g proteose peptone, 13.5 g digestibility serum powder, 5.0 g yeast extract, 2.2 g beef extract, 1.2 g beef liver extract powder, 3.0 g glucose, 2.5 g KH_2PO_4, 3.0 g NaCl, 5.0 g soluble starch, 0.3 g L-cysteine hydrochloride, and 0.3 g sodium thioglycolate, and 1000 mL distilled water, then the pH was adjusted to 7.3 before autoclaving at 121 °C for 20 min. The anaeropack rectangular jars were purchased from Mitsubishi Gas Chemical Company INC (Japan).

2.2. Bacterial isolation

Four grams of freshly voided fecal sample from a healthy female volunteer was homogenized and suspended in 20 mL of sterile physiological saline. The mixture was centrifuged at $2000 \times g$ for 10 min and the suspension was used as human intestinal bacterial mixture. The sample of bacterial mixture was directly used to experiment. The bacterial mixture was serially diluted in water and the dilutions were spread on GAM agar plates. The plates were incubated in anaerobic jars under anaerobic condition at 37 °C for 48 h (Hur et al., 2000). About one hundred different types of bacterial colonies developed on plates.

2.3. Bacterial incubation and sample preparation

Each bacterium was picked up from GAM agar plate and inoculated into 1.0 mL GAM broth, the cultures were anaerobically incubated at 37 °C for 24 h. A 0.1 mL of portion of the preculture bacteria was inoculated into 0.9 mL of GAM broth with 0.1 mM hyperoside. After 48 h of incubation, 100 samples were extracted three times with ethyl acetate of 1.5 of volume of the culture. The ethyl acetate layer was dried and dissolved in 0.3 mL methanol (Minamida et al., 2006). Then, the samples were centrifuged at $12{,}000 \times g$ for 10 min. The supernatant was used for UPLC/MS.

2.4. Ultra-performance liquid chromatographic condition

The liquid chromatography was carried out on a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA). A Syncronis C 18 column (100×2.1 mm i.d., 1.7 μm; Thermo, USA) was carried out for separation. The column was eluted with a gradient mobile phase of acetonitrile (solvent system A) and 0.1% formic acid in ultra-pure water (solvent system B): 0–7.5 min, linear from 10% to 40% A; 7.5–9 min, linear from 40% to 90% A; 9–10 min, held at 90% A for 1 min; 10–11 min, 10% A for equilibration of the column. The flow rate was 0.4 mL/min. The column temperature was maintained at 35 °C, and the autosampler temperature was set at 4 °C.

2.5. Mass spectrometric conditions

A Waters ACQUITY™ Synapt mass spectrometer (Waters Corp., Manchester, UK) was connected to the UPLC system via an electrospray ionization (ESI) interface. The ESI source can be operated in negative mode and the mass selective detector MS can be operated in SIM. The MS parameters were as follows: source temperature 120 °C, desolvation temperature 350 °C, cone voltage 40 V, capillary voltage 3.0 KV, the gas (N_2) flows of cone and desolvation were 50 and 600 L/h. Leucine–enkephalin was used as the lock mass to generate an $[M-H]^-$ ion (m/z 554.2615). In addition, the MS^E experiment carried out for obtaining the parent and fragment mass information of the compounds was performed two scan function, function 1: m/z 100–1000 mass-scan range, 0.5 s scan time, 0.02 s inter-scan delay, 6 V collision energy; function 2: m/z 50–1000, 0.3 s scan time, 0.02 s inter-scan