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Application of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry to determine the metabolites of orientin produced by human intestinal bacteria

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ABSTRACT

An ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) with MetaboLynx™ software combined with mass defect filtering (MDF) method were provided for orientin metabolism study. The chromatographic separation was performed on a 1.7 μm particle size Syncronis C_{18} column using gradient elution system. The components were identified and confirmed according to the mass spectrometric fragmentation mechanisms, MS/MS fragment ions and relevant literature by means of electrospray ionization mass spectrometry in negative ion mode. With this method, a total of three metabolites were identified based on retention time and MS/MS data. The results illustrated that deglycosylation, dehydroxylation and acetylation were the major metabolic pathways of orientin *in vitro* by human intestinal bacteria. Additionally, colonic bacteria were screened for bacteria involved in the conversion of orientin. A gram-negative anaerobic bacterium, strain 45, was newly isolated from healthy human feces. This strain, which was able to cleave the C-glycoside of orientin to produce luteolin and generate some other metabolites, had the similarity of 95.44% with *Enterococcus casseliflavus* and was named *Enterococcus sp.* 45 based on 16S rRNA sequence analysis. In this paper, the metabolic routes, metabolites of oreintin produced by the intestinal bacteria and the *Enterococcus sp.* 45 were investigated for the first time.

1. Introduction

Herba lophatheri is the dried stem and leaf of *Lophatherum gracile* Brongn from the family of *Gramineae*. According to Chinese medicinal theory, *H. lophatheri* affects the body by clearing away heat and reducing fire. It also has a relaxing effect and acts as a diuretic [1]. It is not only used to cure fever, urodynia, and buccal ulceration in clinical use, but it is also more and more commonly served as a tea drink. The main constituents of *H. lophatheri* include phenolic acids, flavonoid glycosides, coumarin, terpenoid, and polyose. The known flavonoid glycosides include orientin, isoorientin, swertiajaponin, vitexin, isovitexin, and luteolin-7-O-β-D-glucoside, which are the major pharmacological active constituents in *H. lophatheri* [2,3]. As a main active compound, orientin demonstrated antiviral, antimicrobial, antioxidant, radioprotective activities [4,5]. As we know, most traditional medicines are administered orally and components of these medicines inevitably come into contact with intestinal microflora in the alimentary tract and then can be transformed by intestinal bacteria before being absorbed from the gastrointestinal tract [6,7]. The bioavailability of flavonoids has been investigated intensively during recent years [8–10]. According to their low plasma concentrations, the systemic availability of intact flavonoids appears to be very limited. The low bioavailability could be due to low absorption or extensive metabolism of ingested flavonoids. Besides undergoing conversion by various enzymes, flavonoids reaching the intestine might be transformed by gut microbiota. Intestinal bacteria have a number of deconjugating enzyme activities, *e.g.* β-D-glucuronidases, β-D-glucosidases and α-L-rhamnosidases, which release aglycones of flavonoids from their glycosides and glucuronides [11–15]. Chinese herbs containing a variety of flavonoid glycosides are generally prepared by boiling them with water in which flavonoid glycosides are quite soluble but flavonoid glycosides are too polar to be absorbed by the intestine. Nevertheless, when the glycosides migrated down to the large intestine, the bacteria residing in the colon could release enzymes to cleave the sugar moieties to transform the glycosides into aglycones which were less polar and became more

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absorbable. More importantly, the bacterial metabolites, which possibly exerted biological activities different from those of the original flavonoids, might be absorbed and further metabolized in the human body. Therefore, it is essential to study their conversions by intestinal bacteria and to identify and characterize the metabolites.

Regarding orientin, its deglycosylation to luteolin, which was a major metabolite by intestinal bacteria, was quite meaningful because luteolin exerted different biological activities from orientin such as antioxidant, anti-inflammatory, antimicrobial and anticancer activities [16]. However, orientin has been studied extensively on the aspect of pharmacological activities rather than the degradation of orientin by human intestinal bacteria.

In this work, we attempted to isolate different pure strains of bacteria from healthy human feces and carried out research on their abilities and characteristics in the metabolism of orientin. Among hundreds of the isolated bacteria, strain 45 was outstanding because of its conversion of C-glucoside *in vitro*, which could result in aglycones and further metabolites whose biological activities differed from that of parent compound. In general, C-glycosides are more resistant toward acid, alkaline and enzymatic treatment than the corresponding *O*-glycosides and little was known about luteolin-producing intestinal bacteria [17]. In this study, we isolated a novel species of orientin conversion bacteria (strain 45) from the feces of healthy human and characterized the genetic properties of the species consequently.

2. Experimental

2.1. Chemicals and reagents

AnaeroPack rectangular jars were purchased from Mitsubishi gas chemical company INC (Japan). Orientin standard substance (purity 99.35%) was purchased from Shanghai Winherb Medical S&T Development Co. Ltd (Shanghai, China). The HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, USA). Formic acid was obtained from Merck KGaA (Darmstadt, Germany). The distilled water was purified by an EPED super purification system (Nanjing, China). Other reagents were of analytical grade.

2.2. Preparation of the general anaerobic medium

The general anaerobic medium (GAM) was prepared 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 with NaOH aqueous solution before adjusting autoclaving to a total volume of 1000 mL. The obtained anaerobic medium was then autoclaved at 121 °C for 20 min.

2.3. Bacterial isolation

Fresh human fecal samples were obtained from a healthy female volunteer who had not taken any medicine in three months and avoided alcohol and food rich in polyphenols in 48 h before fecal collection. 4.0 g fecal sample was weighted and suspended in a centrifuge tube mixed with 20 mL sterile physiological saline, then was homogenized adequately by a vortex-mixer. The mixture was centrifuged at 2000 × *g* for 10 min and the suspension was used as human intestinal bacterial mixture.

The bacterial mixture was diluted serially in sterile water and each of the dilutions was spread on GAM agar plates. The plates were incubated in anaerobic jars under anaerobic condition at 37 °C for 48 h. About one hundred different types of bacterial colonies which developed on plates were picked up [18].

2.4. Preparation of standard solution of orientin

The standard solution of orientin was prepared by dissolving accurately weighed orientin in MeOH to give a final concentration of 5.0 mg/mL. The solution was stored in a refrigerator at 4 °C before analysis.

2.5. Preparation of sample solutions for UPLC-Q-TOF/MS

0.1 mL of about one hundred different bacterial colonies were inoculated into 0.9 mL of GAM broth containing 0.1 mM orientin, respectively, and the media were incubated under anaerobic conditions at 37 °C for 2 days. After the incubation, the incubated solution was extracted with 1.5 times the volume of ethyl acetate three times. Upper organic phase was separated and dried at 50 °C. The residues were dissolved in 0.2 mL MeOH, centrifuged at 13,000 × *g* for 10 min, and the supernatant were analyzed by UPLC-Q-TOF/MS.

2.6. UPLC and MS conditions

Analysis was performed using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with a conditioned autosampler at 4 °C. The separation was carried out on a Syncronis C 18 column (100 mm × 2.1 mm i.d., 1.7 μm; Thermo, USA). The column temperature was maintained at 35 °C. 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 and injection volume was 10 μL. 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 was operated in negative ionization mode with the capillary voltage at 2.0 kV. Source and desolvation temperatures were set at 120 and 350 °C, respectively. The cone and desolvation gas flows were 50 and 600 L/h, respectively. Leucine-enkephalin was used as the lock mass generating an $[M-H]^-$ ion (*m*/*z* 554.2615) to ensure accuracy during MS analysis. The lockspray interval was set at 10 s, and data were averaged over 5 scans. All data collected in centroid mode were acquired using Masslynx™ NT4.1 software (Waters Corp., Milford, MA USA).

2.7. 16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was extracted from the isolate using a Karroten genomic DNA purification kit (Karroten, China). The 16S rRNA sequence of strain was amplified by employing two universal primes, 16S-1F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 16S-1R (5′-AGA AAG GAG GTG ATC C-3′). The polymerase chain reaction (PCR) program used for amplification was as follows: 94 °C for 1 min, followed by 29 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a single final extension step consisting of 72 °C for 10 min. The PCR product was purified from the agarose gel using a Karroten gel purification kit. Sequencing of the 16S rDNA fragments was performed by Majorbio (Shanghai). EzBioCloud performed the homology search of 16S rRNA gene sequence. A phylogenetic tree was constructed using the neighbor-joining method using the CLUSTAL W program and MEGA (ver 5.0) software.