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Ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry for determination of avicularin metabolites produced by a human intestinal bacterium

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ABSTRACT

Intestinal bacteria from human were screened to isolate the specific bacteria involved in the metabolism of avicularin. A Gram-positive anaerobic bacterium, strain 46, capable of metabolizing avicularin (quercetin-3-*O*-arabinoside) was isolated for the first time. Its 16S rRNA gene sequence showed 99% similarity with that of *Bacillus*. Then strain 46 was identified as a species of the genus *Bacillus*, and was named to be *Bacillus* sp. 46. Additionally, the metabolites were analyzed by ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS) technique combined with Metabolynx™ software. The structure of these metabolites were proposed and confirmed by comparing the UPLC retention time and MS/MS spectrum with that of authentic standards. Parent compound and six metabolites were detected in the isolated bacterial samples compared with blank samples. Avicularin (M1) was anaerobic metabolized to its aglycone quercetin (M2) and methoxylated avicularin (M3, M4), then quercetin was converted to quercetin glycosides: quercetin-3-*O*-rhamnoside (M5), quercetin-3-*O*-glucoside (M6) and quercetin-7-*O*-glucoside (M7) by *Bacillus* sp. 46. The metabolic pathway and metabolites of avicularin by the intestinal bacterium *Bacillus* sp. 46 were reported for the first time.

1. Introduction

Quercetin is the most common flavonoid that occurs in many plants and is always conjugated with various sugar moieties to form different quercetin glycosides such as quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rhamnoside (quercitrin), quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-rutinoside (rutin). These quercetin glycosides are widely distributed in plants such as *Hypericum perforatum*, *Himalayan Rheum nobile* and *Saururus chinensis* which are used in traditional medicine for thousands of years [1–6], and have been reported to have many biological activities, such as antioxidant, antidepressant and antibacterial effects [2,3,7,8]. Avicularin (Quercetin-3-*O*-arabinoside) was isolated from *Polygonum aviculare* L, *Himalayan R. nobile*, *H. perforatum* L and *parasitic loranthus* [3,5,9,10]. Avicularin had antioxidant activity by protecting H9c2 cardiomyocytes against oxidative stress-induced cell death and hepatoprotective activity against injury by nitrofurantoin and t-BHP in HepG2 cells [11–13]. Avicularin was also used as urease and fatty acid synthase inhibitors and exhibited anti-inflammatory and anti-diabetic activities [10,14,15].

A major part of the quercetin glycosides were expected to reach the colon after oral administration and were deglycosylated by intestinal bacteria before absorption across the intestine. *Enterococcus casseliflavus* that could metabolize quercetin-3-*O*-glucoside to quercetin was isolated from human [16]. *Fusobacterium* K-60 that metabolized quercitrin to quercetin was also screened from human [17]. In addition, *Eubacterium ramulus* was capable of degrading the aromatic ring systems of rutin and quercetin-3-*O*-glucoside to quercetin and 3, 4-dihydroxyphenylacetic acid [18,19]. Some studies reported that aglycone quercetin exhibited stronger antimicrobial and antioxidant activities than its glycosides [20,21]. Quercitrin metabolites 3, 4-dihydroxyphenylacetic acid and 4-hydroxylphenylacetic acid produced by intestinal bacteria were also found to show more potent anti-platelet aggregation and cytotoxicity against tumor cell lines than quercitrin [22]. In addition, the prevention of H_2O_2-induced apoptosis and anti-inflammatory effects of rutin and quercitrin involved releasing of quercetin [23,24]. Hence we could conclude that the metabolism of avicularin by human intestinal bacteria also affected its

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biological activities. Furthermore, it was reported that the absorption rate and bioavailability of quercetin glycosides were lower than that of aglycone quercetin [25,26]. Thus, intestinal bacteria played an important role in the metabolism and bioavailability of natural components especially flavonoid.

Till now, the metabolism of avicularin by intestinal bacteria has not been reported. In this study, we isolated and characterized the species of a human intestinal bacterium which could metabolize avicularin to various metabolites. To further clarify the metabolic profile of avicularin, ultra performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/Q-TOF-MS) with automated data analysis (MetaboLynx^{TM}) was utilized. UPLC has better chromatographic separations with higher sensitivity and resolution in a much faster time frame than HPLC. In addition, quadrupole time-of-flight mass spectrometry not only provides accurate masses of ions, but also gives valuable structural information from the MS/MS spectra [27,28]. Hence, the UPLC/Q-TOF-MS method could be used to study the multiple bioactive metabolites *in vitro* with minimal operator intervention, reduce data interpretation time and produce high-quality structural information efficiently. The results probably provided helpful information for further *in vivo* metabolism and active mechanism research on avicularin.

2. Experimental

2.1. Instrumentation

Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters, Corp., Milford, MA, USA), consisting of a binary pump solvent management system, an online degasser, and an autosampler. An ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) was applied for all analyses. Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source (ESI). Waters Metabolynx^{TM} (v4.1) program (Waters Corp., Milford, MA, USA) was used to control the UPLC–MS/MS system and for the data acquisition and processing.

2.2. Chromatographic conditions

The UPLC 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.

2.3. Mass spectrometric conditions

The ESI source can be operated in negative mode and optimized conditions for maximum detection of metabolites 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, respectively. Leucine-enkephalin was used as the lock mass to generate an $[M-H]^-$ ion (m/z 554.2615).

2.4. Materials and reagents

Standards for avicularin, quercetin, quercetin-3-*O*-glucoside, quercetion-7-*O*-glucoside and quercetin-3-*O*-rhamnoside were purchased from Shanghai Winherb Medical S & T Development Co. Ltd. (Shanghai Winherb Medical S & T Development Co. Ltd., Shanghai, China). UPLC grade acetonitrile and methanol were purchased from Tedia Company (Tedia Company, Fairfield, USA); Formic acid was obtained from Merck KGaA (Merck KGaA, Darmstadt, Germany); Water from an EPED super purification system (Nanjing EPED Technology Development Co. Ltd., Nanjing, China) was used for UPLC and medium preparation.

2.5. Bacterial isolation and culture conditions

Four grams of human fecal sample from a healthy individual were mixed with 20 mL of sterile physiological saline and then vortex-mixed for 3 min. After centrifugation for 10 min at 2000 × *g*, the suspension was used as human intestinal bacterial mixture which was serially diluted in water and aliquot was plated on general anaerobic medium (GAM) agar plates. These plates were incubated under anaerobic condition at 37 °C for 48 h using the anaeropack rectangular jars which were purchased from Mitsubishi Gas Chemical Company INC (Japan). The GAM contained the following compounds per liter: 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. The pH was adjusted to 7.3 before autoclaving at 121 °C for 20 min. About 100 different bacterial colonies were isolated from the plates after 48 h.

2.6. Screening bacteria for the metabolism of avicularin

Bacteria isolated from the human intestinal tract were assayed for their ability to metabolize avicularin. Separated bacterial colonies were transferred into 1 mL GAM broth, and then cultured anaerobically at 37 °C for 24 h. Each precultured bacterial strain (0.1 mL) was inoculated into 0.9 mL of GAM broth containing 0.1 mM avicularin which was dissolved in methanol. This concentration did not have any effect on cell growth. After 48 h of anaerobic incubation, 100 culture samples were extracted three times with ethyl acetate of 1.5 volume of the culture. The ethyl acetate layer was dried and dissolved in 0.3 mL methanol [29]. Then the samples were centrifuged at 12,000 × *g* for 10 min. Each supernatant was injected into UPLC/MS. One strain of bacteria was found to metabolize avicularin and was further to study.

2.7. 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA of strain 46 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-2F: (CATGCAAGTCGARCG), 16S-2R: (GGTGTGACGGGCGGT). 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 rRNA fragments was performed by Majorbio (Shanghai, China). The homology search of 16S rRNA gene sequence was performed by EzBioCloud server. A phylogenetic tree was constructed using the neighbor-joining method of the CLUSTAL W program and MEGA (ver 5.0) software [30].

3. Results and discussion

3.1. Characterization of a human intestinal bacterium

The 16S rRNA gene of the isolated bacterium was 1264 base pair sequences, and the nucleotide sequence has been registered in GenBank under accession No. KC257405. After compared the