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Biotransformation of the SDG in defatted flaxseed into END co-cultured by three single bacterial colonies

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ABSTRACT

Enterodiol (END) possesses the estrogenic and antiestrogenic activities, which could prevent the development of breast cancer and prostate cancer, as well as menopausal syndrome.

Previous studies in our laboratory set up a bio-transformation method for largely yielding secoisolariciresinol (SECO) from the substrate of defatted flaxseeds by strain *Bacteroides uniformis* ZL-I. In this research, another two single colonies, designated as strain ZL-II and strain ZL-III, were isolated, which were closely related to *Eubacterium limosum* species (ZL-II), and *Eggerthella lenta* species (ZL-III) on the basis of 16S rRNA gene sequence data. Under the combining actions of strains ZL-(I + II + III), END could be produced from defatted flaxseed directly, ZL-II was proved to possess the activities of demethylation, while ZL-III had the activities of dehydroxylation. Secoisolariciresinol diglucoside (SDG) existed in the form of oligomeric with 3-hydroxy-3-methyl glutaric acid in flaxseed could be efficiently transformed into END under the co-culture of strains ZL-(I + II + III), with the conversion rate of more than 90%. The method for mass-producing END from defatted flaxseed reported here is meaningful not only for the medicinal values of END but also for the resource utilization of flaxseed materials.

1. Introduction

Phytoestrogens are kinds of estrogen-like active compounds that usually presented in plants, such as the isoflavonoides in soy beans and lignans in flaxseed [1]. Enterodiol (END) is one of the lignan-type phytoestrogens, which had potent antiestrogenic effects on estrogen receptor-positive breast cancer, it can modulate the immune response by acting on nuclear factor-κB (NF-κB) signaling, which in turn resulted in decreased tumor necrosis factor-α (TNF-α) production [2,3]. In addition, END had antioxidant activity [4,5]. As known, END was mainly found from the biosamples of mammalians [6,7], it was proved that many lignanoid constituents in plant resources, such as secoisolariciresinol diglucoside (SDG), matairesinol [8], lariciresinol, isolariciresinol [9,10], 7-hydroxymatairesinol [11], pinoresinol, arctigenin, syringaresinol [12] and asarinin [13], could act as the precursors of END *in vivo*, and they could be transformed to END under the biological transformation of human intestinal bacteria. Flaxseed is the known richest resource of lignans, which contains approximately 6.1–11.3 mg g^{-1} SDG in dried mass [14]. However, the SDG in flaxseed was in the form of oligomeric structure composed of five SDG residues interconnected by four 3-hydroxy-3-methyl glutaric acid (HMGA) residues [15], which brought difficulties for the isolation of SDG from flaxseed by using the traditional chemical methods. Therefore, we tried to directly transform the SDG-HMGA residues in flaxseed into END by human intestinal bacteria *in vitro*, the method of microbial transformation has been proved workable [15,16]. As the single strain clones which were in charge of the whole catalytic reaction process among the microbiota were still unknown, in this study, three colonies with different morphology were isolated and identified from the active microbiota after repetitive subculturing and plate cultiviation, the END producing technology by taking defatted flaxseed as substrate which was co-cultured with the three colonies was also investigated.

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2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile was purchased from Merck KGaA Co. Ltd. (Darmstadt, German) and purified water was provided by Hangzhou Wahaha Co. Ltd. (Zhejiang, China). Analytical-grade methanol, *n*-butanol, petroleum ether, ethanol, KH_2PO_4 and K_2HPO_4 were purchased from Beijing Chemical Reagents Co. Ltd. (Beijing, China). SECO and END used as standards were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Amberlite XAD-2 macroporous resin (20–60 mesh size, 330 $m^2\ g^{-1}$ average surface area) was purchased from Sigma–Aldrich Co. Ltd (Bellefonte, USA). Optical rotations were measured in MeOH solutions with an Autopol III automatic polarimeter (Rudolph Research Analytical, USA) at 25 °C. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA-500 spectrometer.

SDG was extracted from defatted flaxseed according to the references [17–19]. The purified compound was identified by ^1H and ^{13}C NMR, which were same to those of SDG [20,21], the configuration of it was confirmed as (+)-SDG by polarimetry $[\alpha]_D^{25}$ +3.6 (c = 0.25, MeOH) [20,22]. END standard was purchased from Sigma–Aldrich Co. Ltd. (St. Louis, MO., USA).

2.2. Plant materials

Flaxseeds were collected from Bei-An County of Heilongjiang Province, China, and were identified as the dried seeds of *Linum usitatissimum* L. Voucher specimens (sample No. 071024) were deposited in the herbarium of pharmacognosy research group, School of Pharmaceutical Sciences, Peking University. They were ground into powder (pass 40 mesh sieve) and then defatted by petroleum ether prior to use.

2.3. Collection and processing of fecal samples

Initially, fresh fecal specimens (*ca.* 4.0 g each), obtained from 10 healthy young subjects (four females and six males, 21–30 years old), were suspended in 20 ml sterile phosphate buffer saline (PBS, 2.6 g L^{-1} KH_2PO_4, 1.85 g L^{-1} K_2HPO_4, pH 7.4) and 2 ml such fecal suspension was transferred to 20 ml culture medium (see below).

2.4. Culture media and bacterial culture

Anaerobic broth and anaerobic agar were purchased from Beijing Land Bridge Technology Co. Ltd. (Beijing, China). Anaerobic Broth (g L^{-1}) consisted of peptone 15.0, yeast 5.0, soybean peptone 5.0, beef powder 5.0, glucose 5.0, sodium chloride 5.0, soluble starch 3.0, cysteine 0.5, potassium dihydrogen phosphate 2.5, hemin 0.005, and vitamin K 1 0.001; anaerobic agar was anaerobic broth plus agar 15.0 g L^{-1}. Carbon-free medium contained (in one liter) NaCl 3 g, KH_2PO_4 2.6 g, K_2HPO_4 1.85 g, 1% (v/v) reducing solution (30 g L^{-1} L-aminothiopropionic acid and 30 g L^{-1} sodium hyposulfite, dissolved in PBS), and 1 g NH_4Cl.

Bacterial culture procedure was conducted as follows: 0.5 g of defatted flaxseeds (as substrate and as carbon source) was added into each of tubes containing 5 ml of carbon-free medium, then sealed with liquid paraffin and autoclaved at 121 °C for 15 min. Into the culture system, 2 ml fecal suspension was added, and the incubation was conducted at 37 °C for 72 h.

Supernatant of the culture was analyzed for the appearance of END by HPLC.

2.5. Isolation of END-producing bacteria

The bacterial culture, possessing transforming activity, was serially diluted (10^{-1} to 10^{-6}) and seeded on anaerobic agar plates and anaerobically incubated for 24 h at 37 °C. Well isolated single colonies from the agar plates spread with the 10^{-5} and 10^{-6} dilutions were picked and cultured in anaerobic broth, and then tested their ability to convert SDG into END.

2.6. Sequencing of the bacterial 16S rRNA gene

The target bacterium was incubated at 37 °C in anaerobic medium for 24 h and collected by centrifugation at 12,000 g for 2 min. Total DNAs were extracted with a TIANamp Bacteria DNA Kit (TIANGEN, Beijing) following the manufacturer's protocol. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with primers BSF (AGAGTTTGATCCTGGCTCAG) and BSR (AAGGAGGTGATCCAGCCGCA). Amplification proceeded in a reaction mixture containing 0.2 mM of each primer, 25 μl of 2× Taq PCR Master Mix (TIANGEN, Beijing), and 21 μl of template DNA. The PCR program was as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min; a final extension step at 72 °C for 5 min. PCR products were sent to Sangon (Beijing, China) for sequencing.

2.7. High-performance liquid chromatography (HPLC)

The HPLC system consisted of Agilent 1200 series HPLC apparatus (Agilent Technologies, USA), including high-pressure binary-gradient solvent-delivery pump, DAD detector, autosampler, thermostat column compartment and chemstation (9.01 edition). Zorbax SB-C_{18} column (4.6 mm × 250 mm, 5 μm) was used to analyze all of the samples. Mobile phase consisted of water (A) and acetonitrile (B) in a linear gradient change from 100% A to 50% A and 50% B in 30 min. Detection wavelength was 280 nm, and the temperature of the column oven was 25 °C with a flow rate of 1.0 ml min^{-1}.

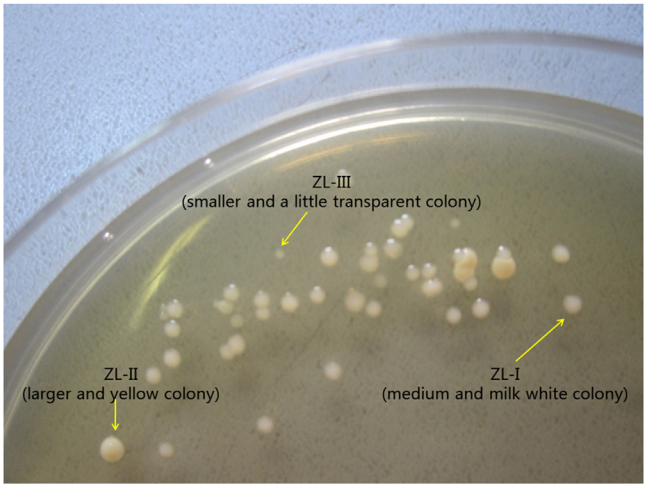

Fig. 1. The characteristic colony morphological features of strains ZL-I, ZL-II and ZL-III cultured on anaerobic agar plate.

2.8. Calibration of the SDG and END curves

The reference stock solutions of SDG (200.0 μg ml^{-1}) and END (208.0 μg ml^{-1}) were dissolved in methanol. A series of solutions of SDG (2.5–200.0 μg ml^{-1}) and END (1.30–208.0 μg ml^{-1}) were prepared by diluting the stock solutions with methanol. The calibration equations were obtained by plotting HPLC peak areas (Y) *versus* the concentrations of calibrators (X, mg ml^{-1}), which were as follows: $Y = 10.25X - 5.892$ ($R^2 = 0.9990$) for SDG, with a good linearity over the range from 2.5 to 200 μg ml^{-1}; $Y = 9668X + 13.17$ ($R^2 = 0.9990$) for END, with a good linearity over the range from 1.30 to 208.0 μg ml^{-1}.

2.9. Limits of detection and quantification

Stock solutions of SDG and END standards were separately diluted to make a series of solutions with methanol and analyzed by HPLC. On the basis of signal-to-noise ratio (S/N), the limits of detection (LOD, $S/N = 3$) were determined to be 1.25 μg ml^{-1} (SDG) and 0.33 μg ml^{-1} (END); the limits of quantification (LOQ, S/N = 10) were 2.5 μg ml^{-1} for SDG and 1.3 μg ml^{-1} for END.

2.10. Determination of SDG contents in defatted flaxseed and fermented defatted flaxseed residue

Extraction of SDG from defatted flaxseed was carried out basically according to the method described by Zhang [23]. Briefly, portions (1.0 g) of defatted flaxseed were extracted with 15 ml of 50% ethanol–water (v/v) in test tubes by sonication (ultrasonic power: 200 W) for 30 min in a 40 °C water bath. After centrifugation (3000 rpm, 15 min), the pellet was extracted again by the same method. The supernatants were merged together and subjected to alkaline hydrolysis for 3 h using 0.25 M sodium hydroxide at room temperature. After hydrolysis, the samples were acidified to pH 4–6 using 6 M hydrochloric acid, and their volume was adjusted to 50 ml in volumetric flasks.

2.11. Sampling of the cultures

A volume of 200 μl of culture was sampled every 24 h and extracted with 400 μl *n*-butanol saturated with water. A portion of *n*-butanol extracts (320 μl) was transferred to a centrifuge tube and evaporated to dryness by N_2. The residue was dissolved in 200 μl methanol and centrifuged for 3 min (10,000 rpm min^{-1}), and then 20 μl of the supernatant was filtered and analyzed by HPLC.

3. Results

3.1. Selection and characterization of END-producing bacteria

As shown in Fig. 1, more than 50 colonies could be differentiated from the culture plate, all of them were picked up in the beginning. But it was found later that the colonies could be divided into three categories basing on their morphological features, which were