Enantioselective Oxidation of Enterodiol to Enterolactone by Human Intestinal Bacteria

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Received May 8, 2007; accepted July 26, 2007

In the course of our experiments on the metabolic conversion of lignans to the estrogenic substances enterodiol (END) and enterolactone (ENL) by human intestinal flora, we isolated two anaerobes, *Ruminococcus* sp. END-1 and strain END-2, capable of oxidizing END. The former selectively converted (–)-END to (–)-ENL, while the latter selectively converted (+)-END to (+)-ENL, indicating enantioselective oxidation by intestinal bacteria.

Key words human intestinal bacteria; oxidation; enantioselectivity; enterodiol; enterolactone

Plant lignans, such as secoisolariciresinol diglucoside (SDG), pinoresinol diglucoside (PDG), arctiin, and matairesinol, are converted to the mammalian lignans enterolactone (ENL) and enterodiol (END) by intestinal bacteria.^{1–6} The metabolic processes of the aforementioned intestinal bacteria include deglucosylation, ring cleavage, demethylation, dehydroxylation, and oxidation. We have isolated bacteria that participate in these metabolic processes from human feces.^{5–8} ENL and END have two enantiomeric mirror image forms (Fig. 1). A (–)-form of ENL was formed by anaerobic incubation of PDG derived from *Eucommia ulmoides* OLIV. with a bacterial mixture from human feces.⁵ On the other hand, a (+)-form of ENL was obtained by incubation of SDG isolated from flaxseed.⁶ We were interested in whether the same or different bacteria are involved in the biotransformation of the respective enantiomeric forms. Previously, we isolated *Eggerthella* (*Eg.*) sp. SDG-2 and strain ARC-1⁷ and demonstrated that these two bacteria participated in enantioselective dehydroxylation when incubated with dihydroxyenterolactone (DHENL) or dihydroxyenterodiol (DHEND), *i.e.*, *Eg.* sp. SDG-2 converted (–)-DHENL and (+)-DHEND to (–)-ENL and (+)-END, respectively, but not for (+)-DHENL and (–)-DHEND. Conversely, strain ARC-1 had dehydroxylation activity for (+)-DHENL and (–)-DHEND, but not (–)-DHENL and (+)-DHEND. We also reported the isolation and characterization of a human intestinal bacterium, *Eubacterium* (*E.*) sp. ARC-2, capable of demethylating (–)-arctigenin to (–)-DHENL.⁸ *E.* sp. ARC-2 could convert both (+)-secoisolariciresinol and (–)-secoisolariciresinol 4''-*O*-methyl ether to (+)-DHEND and (–)-DHEND, respectively, without enantioselectivity.

In the course of our studies on the enantioselective biotransformation of natural lignans to mammalian lignans, we

report here the isolation of bacteria capable of enantioselectively oxidizing (+)- and (–)-END from human feces.

MATERIALS AND METHODS

General An anaerobic incubator, model EAN-140 (Tabai Co., Osaka, Japan), was used for the incubation of fecal suspensions and intestinal bacteria. Optical rotations were measured in MeOH solutions with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan). ¹H- and ¹³C-NMR, ¹H–¹H-correlated spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC), heteronuclear multiple-bond coherence (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiments were performed on Varian Unity 500 (¹H: 500 MHz, ¹³C: 125 MHz) and Varian Gemini 300 (¹H: 300 MHz, ¹³C: 75 MHz). Thin-layer chromatography (TLC) was carried out on silica gel precoated 60 F₂₅₄ plates (0.25 mm, Merck Co., Darmstadt, Germany), and spots were detected under a UV lamp or exposing I₂ vapor. Silica gel BW-820 MH (Fuji Silysia, Aichi, Japan) was used for column chromatography. The DNA sequences were aligned using DNASIS version 3.0 (Hitachi Software Engineering Co., Tokyo, Japan).

Chemicals and Media General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan). Brain heart infusion (BHI) broth was purchased from Beckton Dickinson (Sparks, MD, U.S.A.). (–)-END was prepared by anaerobic incubation of (–)-secoisolariciresinol 4''-*O*-methyl ether with a human intestinal bacterial (HIB) mixture.⁷ (+)-END was obtained from SDG by incubation with an HIB mixture.⁶ A racemic mixture of ENL was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, U.S.A.).

LC/MS Analysis HPLC/MS was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) with a photodiode array detector and an Agilent 1100 series binary pump, and an Esquire 3000plus mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) coupled with an ESI interface and an ion trap mass analyzer, under the following conditions: column, chiral CD-Ph (Shiseido, Tokyo, Japan, 4.6 mm×150 mm); mobile phase, 0.1% TFA (solvent system A) and CH₃CN (solvent system B) in gradient modes (B from 30 to 45% for 30 min); flow rate,

Fig. 1. Bacterial Transformation of Enterodiol to Enterolactone

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Fig. 2. Dendrograms of Phylogenetic Affiliation of *R. sp. END-1* (A) and Strain END-2 (B)

The sequences of *R. sp. END-1* and strain END-2 were compared with those from the Genbank database with the ClustalW program.

0.5 ml/min; detection, UV at 280 nm; and temperature, 30 °C. High-purity nitrogen was used as dry gas at a flow rate of 10 l/min, with temperature of 360 °C. Helium was used as nebulizer at 50 psi. The electrospray ionization (ESI) interface and mass spectrometric parameter were optimized to obtain maximum sensitivity.

Isolation of an HIB Strain Capable of Oxidizing (–)-END to (–)-ENL A bacterial suspension from the fresh feces of a healthy volunteer was repeatedly cultured in 2 ml of GAM broth containing 0.1 mM (–)-END as a substrate at 37 °C in an anaerobic incubator. A portion of the culture with metabolic activity was seeded on GAM agar plates and anaerobically incubated for 48 h at 37 °C. Colonies were selected and screened for their activity in transforming (–)-END to (–)-ENL. This procedure was repeated until a single strain with oxidizing activity was isolated.

Isolation of an HIB Strain Capable of Oxidizing (+)-END to (+)-ENL Similarly, a bacterial suspension was repeatedly cultured in 2 ml of GAM broth containing 0.1 mM (+)-END as a substrate under anaerobic conditions at 37 °C. A portion of the culture with metabolic activity was seeded on BHI agar plates and anaerobically incubated for 72 h at 37 °C. Colonies were selected and screened for activity. This procedure was repeated until a single strain was isolated.

Sequencing a Bacterial 16S rRNA Gene A bacterium was incubated at 37 °C in an anaerobic incubator for 2 d, and the cells were collected by centrifugation at 10000×*g* for 10 min. Total DNAs were extracted with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The 16S rRNA gene was amplified using the polymerase chain reaction (PCR) with two forward and reverse primer sets based on those of various strains from the database: Bac 1F (AGAGTTTGATCCTGGCTCAG) and Bac 1R (CCGTATTACCGCGGCTGCTG); and Bac 3F (TAAC-TACGTGCCAGCAGCCG) and Bac 3R (CCCGGGAACG-TATTCACCG). Amplification proceeded in a reaction mixture containing 1 U of KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), 1×PCR buffer mix, 0.8 mM dNTP mix (0.2 mM each), 1 mM MgSO₄, 0.3 μM of each primer, and 100 ng of template DNA. The PCR program was: 94 °C for 2 min, 30 cycles of 94 °C for 14 s, 55 °C for 30 s, 68 °C for 45 s, and finally 68 °C for 5 min. The PCR products were purified using a QIA Quick PCR Purification Kit (Qiagen), and directly sequenced using a Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) with primers (Bac 1F, Bac 1R, Bac 3F, and Bac 3R) and an ABI PRISM 310 (Applied Biosystems).

Incubation of END with Intestinal Bacteria and Preparation of Samples for HPLC A 100 μl portion of precultured *Ruminococcus* (*R.*) *sp. END-1* and strain END-2 was inoculated into 2 ml of GAM broth with 0.2 mM (+)-END and (–)-END, respectively, and the mixture was anaerobically incubated at 37 °C for 120 h. A 100 μl aliquot was removed and extracted three times with 100 μl of ethyl acetate. After evaporation of the ethyl acetate *in vacuo*, the residue was dissolved in 0.3 ml of MeOH. The MeOH solution was filtered through a 0.2 μm membrane filter, and a 10 μl portion was injected onto a chiral column for HPLC analysis under the conditions described above.

RESULTS AND DISCUSSION

A single bacterium (strain END-1) was isolated by repeated incubation of a bacterial mixture of human feces in GAM broth containing 0.1 mM (–)-END, followed by the screening of colonies on GAM agar plates by monitoring the transformational activity of (–)-END to (–)-ENL. To identify the strain, bacterial 16S rRNA gene sequencing was performed. The 16S rRNA gene sequence of strain END-1 (Genbank accession no. EF451052) showed 99% and 98% similarity with *R. sp. M-1* (AB125231) and *R. productus* SECO-Mt75m3 (AY937379), respectively (Fig. 2A). Furthermore, similarity in sequence with those of other *R.* species was greater than 90% showing that the strain may belong to *R.* species based on the 16S rRNA gene sequence, and strain END-1 was tentatively named *R. sp. END-1*.

For studying substrate specificity, the respective enantiomers of END and ENL were analyzed on a chiral column with LC/MS. In the elution profile from the analytical HPLC with a chiral column, a pair of enantiomers showed different retention times (Fig. 3), but the same mass spectral patterns. The retention times of (+)-END and (–)-END were 21.6 and 24.3 min, respectively, while those of (–)-ENL and (+)-ENL were 30.7 and 31.6 min, respectively. When incubated with a mixture of (+)- and (–)-END for 120 h, *R. sp. END-1* converted (–)-END, but not (+)-END (Fig. 3).

Due to the lack of (+)-END conversion, we tried to isolate another bacterial strain capable of oxidizing (+)-END. A single bacterium (strain END-2) was isolated by repeated incubation of a bacterial mixture of human feces in GAM broth containing 0.1 mM (+)-END, followed by the screening of colonies on BHI agar plates. The 16S rRNA gene sequence of strain END-2 (EF451053) showed 96% and 91% similarity with those of the anaerobic bacterium ED-Mt61/PYG-s6

Fig. 3. HPLC Elution Profiles of Enantiomeric Metabolites by *R. sp.* END-1 and Strain END-2

Portions of the culture of *R. sp.* END-1 were collected at 0 h and 120 h. Only (-)-END was converted to (-)-ENL. Similarly, portions of the culture of strain END-2 were collected at 0 h and 120 h. Only (+)-END was converted to (+)-ENL.

(DQ100449) and *R. hydrogenotrophicus* (X95624), respectively (Fig. 2B).

To confirm enantioselective conversion, strain END-2 was incubated with a mixture of (+)- and (-)-END for 120 h. Chiral HPLC analysis showed that only (+)-END was converted to (+)-ENL by strain END-2 (Fig. 3).

In the present study, we isolated two bacteria, *R. sp.* END-1 and strain END-2, capable of oxidizing END to ENL enantioselectively. These results suggest that the lignan metabolism in the human gastrointestinal tract may be greatly influenced by specific bacteria with enantioselective dehydroxylation and oxidation capabilities.

Both bacteria, *R. sp.* END-1 and strain END-2, can be used for selective preparation of the (+)- or (-)-form of ENL from the racemic END.

This is first report on enantioselective oxidation of END by HIB. Studies on substrate specificity to other lignans, the kinetics of the respective bacterial strains in oxidation, and

the mechanism of the oxidations in the anaerobic bacteria are now by progress.

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