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Transformation of trachelogenin, an aglycone of tracheloside from safflower seeds, to phytoestrogenic (−)-enterolactone by human intestinal bacteria

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ABSTRACT

For the purpose of surveying naturally occurring precursors of oestrogenic substances, and their metabolic processes, to mammalian lignans such as enterodiol (END) and enterolactone (ENL), many plant lignans have been studied. Trachelogenin, an aglycone of tracheloside, occurring in the seeds of *Carthamus tinctorius* L. (safflower), was demonstrated to transform to seven metabolites, including (−)-ENL, by anaerobic incubation with a human faecal bacterial mixture, when the reaction was monitored by LC/MS. The structures of the metabolites were determined by spectroscopic means after a large-scale incubation and purification of the respective metabolites. Moreover, the ligand-binding affinity of these metabolites to oestrogen receptors (ERs) α and β was measured in comparison with that of (+)-ENL. (−)- and (+)-ENL were found to significantly bind to both ERα and β, in which an appreciable difference in affinity was observed between (+)- and (−)-ENL for ERβ, but not for ERα.

1. Introduction

Some plant lignans have been demonstrated to be precursors of the mammalian lignans, enterodiol (END) and enterolactone (ENL), which show oestrogenic properties due to the structural similarity and biological activity as isoflavones (Bingham, Atkinson, Liggins, Bluck, & Coward, 1998; Dixon, 2004; Wang, 2002). Since END and ENL were detected in urine of humans, the metabolism of plant lignans as sources of mammalian lignans and structures of their metabolites have been extensively investigated. Several reports have indicated the crucial role of intestinal bacteria in formation of mammalian lignans by using germ-free rats or antimicrobials-pretreated rats, where no mammalian lignans were detected (Bowey, Adlercreutz, & Rowland, 2003; Kilkkinen et al., 2002). Secoisolariciresinol diglucoside (SDG), pinoresinol diglucoside (PDG), arctiin, sesamin, asarinin, matairesinol, 7′-hydroxymatairesinol, lariciresinol, isolarisiresinol, syringaresinol and even dietary lignins were reported to be precursors of mammalian lignans (Begum et al., 2004; Heinonen et al., 2001; Jin & Hattori, 2011; Liu, Saarinen, & Thompson, 2006; Peñalvo, Heinonen, Aura, & Adlercreutz, 2005). However, the bacterial transformation of tracheloside, a major lignan glycoside of seeds of *Carthamus tinctorius* L. (safflower), to mammalian lignans has not yet been examined, though the structure is quite similar to that of arctiin. Tracheloside has an additional hydroxy group at the C-3 position of a tetrahydrofuran moiety of arctiin. Nose, Fujimoto, Takeda, Nishibe, and Ogihara (1992) reported that tracheloside and arctiin were transformed into their aglycones and demethylation products by incubation with rat intestinal bacteria, though they did not detect any oestrogenic compounds such as ENL. However, Xie, Ahn, et al. (2003) demonstrated the conversion of arctiin to ENL by human intestinal bacteria (HIB). Alam et al. (2006) reported that the oil of safflower seeds improved osteoporosis in ovariectomised rats. This may be attributable to tracheloside and its intestinal bacterial metabolites, because phytoestrogens are potentially important in the prevention of postmenopausal osteoporosis, caused by oestrogen deficiency (Draper et al., 1997).

In this paper, we describe the transformation of trachelogenin, an aglycone of tracheloside, to (−)-ENL, and possible metabolic processes, by using an LC/MS technique. Moreover, we report a binding affinity of tracheloside, trachelogenin and their metabolites for two types of oestrogen receptors (ERs).

2. Materials and methods

2.1. General

An anaerobic incubator, EAN-140 (Tabai Co., Osaka, Japan), was used for incubation of faecal suspensions and intestinal bacteria.

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Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan) and electron impact mass spectra with a JMS-GC mate mass spectrometer at an ionisation voltage of 70 eV (Jeol Co., Akishima, Japan). ^{1}H and ^{13}C NMR, ^{1}H–1H-correlated spectroscopy (COSY), heteronuclear multiple quantum coherence, heteronuclear multiple bond coherence (HMBC), and nuclear Overhauser effect spectroscopy experiments were done on Varian UNITY 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) and Varian Gemini 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) NMR spectrometers, using CD_3OD as a solvent. Thin-layer chromatography (TLC) was carried out on silica gel pre-coated 60 F_{254} and RP-18$_{254}$S plates (0.25 mm, Merck Co., Darmstadt, Germany) and spots were detected under a UV lamp or exposing to I_2 vapour. Silica gel BW-820 MH (Fuji Silysia, Aichi, Japan) was used for column chromatography.

2.2. Plant material

Seeds of *C. tinctorius* L. were purchased from Sakata-no-tane Co. (Yokohama, Japan), and a specimen is kept in our laboratory.

2.3. Intestinal bacteria

Eubacterium sp. ARC-2 and *Eggerthella* sp. SDG-2 used in this experiments were previously isolated in our laboratories (Jin et al., 2007a; Wang, Meselhy, Li, Qin, & Hattori, 2000).

2.4. Chemical and media

General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan). Naringenase and 17β-estradiol (E2) were purchased from Sigma Aldrich Japan Co. (Tokyo, Japan). Chromatographic resin DIAION was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). Tracheloside was isolated from the seeds of *C. tinctorius* and trachelogenin was obtained by enzymatic hydrolysis of tracheloside as follows: the softened and ground seeds (2 kg) were extracted three times by immersing in MeOH (10 L) overnight. The combined solutions were concentrated to *ca.* 500 ml under vacuum and then extracted with 300 ml of hexane, followed by evaporation of the solvent to yield a MeOH-soluble residue (148 g), which was chromatographed on a DIAION column (8 cm × 70 cm) eluting with MeOH–H_2O (1:1). Moreover, the fractions including tracheloside were chromatographed on a silica gel column (4 cm × 40 cm), eluting with $CHCl_3$–MeOH (10:1 → 9:1), to yield tracheloside (12 g).

Tracheloside (10 g) was dissolved in 1500 ml of 100 mM acetate buffer (pH 5.4) and incubated with 3 g of naringinase at 37 °C overnight. The mixture was extracted with ethyl acetate and the ethyl acetate-soluble fraction was evaporated to yield a residue, which was further chromatographed on a silica gel column (3 cm × 30 cm), eluting with $CHCl_3$–MeOH (100:1 → 50:1). Then, trachelogenin was obtained in a yield of 3 g. The metabolite was identified by comparing the ^{1}H NMR, ^{13}C NMR, and MS spectra, as well as an optical rotation value with those reported, and the purity was confirmed by HPLC. For this experiment, a 99% pure sample was used as a substrate.

(+)- And (−)-END, (+)- and (−)-ENL, (−)-dihydroxyenterolactone (DHENL), arctiin and arctigenin were prepared by same methods as reported previously (Jin et al., 2007b).

2.5. LC/MS analysis

HPLC 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 Daltonic GmbH, Bremen, Germany), coupled with an electrospray ionisation (ESI) interface and an ion trap mass analyser. Analysis of transformation products was performed under the following conditions: column, TSK-gel ODS-80Ts (Tosoh Co., Tokyo, Japan, 4.6 mm × 150 mm); mobile phase, 0.1% acetic acid (solvent system A) and CH_3CN (solvent system B) in a linear gradient mode (B from 20% to 50% in 30 min); flow rate, 1.0 ml/min; detection, UV 254 nm; temperature, 30 °C. Analysis of (+)- and (−)-ENL was performed under the following conditions: column, chiral CD-Ph (Shiseido, Tokyo, Japan, 4.6 mm × 250 mm); mobile phase, 0.1% acetic acid (solvent system A) and CH_3CN (solvent system B) in linear gradient modes, B from 30% to 48% for 36 min); flow rate, 0.5 ml/min; detection, UV at 280 nm; temperature, 30 °C. The scan range of the ion trap was from *m/z* 50 to 1000 in the positive ion mode. High-purity nitrogen was used as dry gas at a flow rate at 10 L/min, dry temperature at 360 °C. Helium was used as nebuliser at 50 psi. The ESI interface and mass spectrometric parameters were optimised to obtain maximum sensitivity.

2.6. Preparation of an HIB mixture

Fresh faeces (5 g), obtained from a healthy subject, was homogenised in 95 ml of GAM broth and the sediments were removed by decantation to give a 5% HIB mixture.

2.7. Incubation of trachelogenin with an HIB mixture and preparation of samples for HPLC

A 6 ml portion of a 5% HIB mixture was inoculated into 60 ml of GAM broth containing 0.5 mM trachelogenin and anaerobically incubated at 37 °C. A 750 μl aliquot was then taken out at 12 h intervals and extracted three times with 300 μl of ethyl acetate. After evaporation of the ethyl acetate under reduced pressure, the residue was dissolved in 50 μl of MeOH. The MeOH solution was filtered through a 0.2 μm membrane filter, and a 10 μl portion was injected to a column for HPLC analysis under the conditions described above.

2.8. Isolation of trachelogenin metabolites

Eubacterium sp. ARC-2 and *Eggerthella* sp. SDG-2 were taken from GAM agar plates and anaerobically incubated in 100 ml of GAM broth for 48 h at 37 °C. Then, trachelogenin (1 g) and each bacterial suspension (100 ml) of the two strains were added to 0.8 L of GAM broth and incubated at 37 °C in an anaerobic incubator. After confirmation of metabolite formation by TLC, the reaction mixture was then extracted three times with 400 ml of ethyl acetate. The organic layer was evaporated, under reduced pressure, to give a residue. The residue was applied to a column of silica gel (4 cm × 50 cm), which was eluted with a gradient solvent system of $CHCl_3$–MeOH (100:1 → 50:1) to yield metabolites **1**, **4**, **5**, **6**, and **7**. Metabolites **2** and **3** were obtained by preparative HPLC.

2.8.1. (3S,4S)-3-(3,4-Dihydroxybenzyl)-4-(3,4-dimethoxybenzyl)-3-hydroxydihydrofuran-2(3H)-one (1)

Colourless oil. ESI-MS *m/z*: 375 $[M+H]^+$, 392 $[M+H_2O]^+$. This metabolite was identified by comparing the ^{1}H and ^{13}C NMR spectra with those published (Nose et al., 1992).

2.8.2. (3S,4S)-3-(3,4-Dihydroxybenzyl)-3-hydroxy-4-(3-hydroxy-4-methoxybenzyl)-dihydrofuran-2(3H)-one (2):

Colourless oil. ESI-MS *m/z*: 361 $[M+H]^+$, 378 $[M+H_2O]^+$. ^{1}H NMR (CD_3OD, 500 MHz) δ: 2.50–2.85 (3H, m, H-4, H-7″), 2.90 (1H, dd, *J* = 7.0, 14.5 Hz, H_a-7′), 3.03 (1H, dd, *J* = 5.5, 14.5 Hz, H_b-7′), 3.80 (3H, s, –OC$\underline{H}_3$), 3.84 (1H, dd, *J* = 7.5, 8.5 Hz, H_a-5), 4.01 (1H, dd, *J* = 7.5, 8.5 Hz, H_b-5), 6.57 (2H, dd, *J* = 2.0, 8.0 Hz, H-6′, 6″), 6.66 (1H, d, *J* = 2.0 Hz, H-2″), 6.70 (1H, d, *J* = 2.5 Hz, H-2′), 6.74 (1H, d, *J* = 8.0 Hz, H-5′), 6.80 (1H, d, *J* = 8.0 Hz, H-5″).