Purification of Biotransformation Products of Cis-Isoflavan-4-ol by Biphenyl Dioxygenase of Pseudomonas pseudoalcaligenes KF707 Strain Expressed in Escherichia coli

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Abstract

Isoflavone has multiple beneficial effects on human health, especially through its antioxidant and anticancer activities. The biotransformation of isoflavone using byphenyl dioxygenase could be performed to extend the diversity of flavonoids and to improve their biological and physiological properties. Biotransformation of two enantiomers (3R, 4R)-cis-isoflavan-4-ol and (3S, 4S)-cis-isoflavan-4-ol by E. coli JM109 (pJHF108) carrying a biphenyl dioxygenase gene from P. pseudoalcaligenes KF707 produced two products, designated as CM1 and CM2. The products had a retention time of 11.9 and 14.6 min, respectively, and the same absorption peaks at 204, 220, and 275 nm. CM1 and CM2 had [M-H\(_2\)O+H]+ at m/z 225. Based on the molecular mass and hydrolysis products, we proposed that epoxidation occurred on cis-isoflavan-4-ol. Chloroform extraction instead of ethyl acetate extraction was performed to improve the stability of cis metabolites, CM1 and CM2.

Keywords: biotransformation, biphenyl dioxygenase, isoflavone, P. pseudoalcaligenes

1. Introduction

Flavonoids are commonly divided into six subgroups and classified based on the connection position of the B and C rings as well as the degree of saturation, oxidation, and hydroxylation of the C ring [1]. The six subgroups are flavonols, flavones, flavanones, flavan-3-ols (or catechins), isoflavones, and anthocyanidins [1]. Isoflavones are a flavonoid subgroup found in plants and exclusively distributed in leguminose plants such as soybeans [1-3]. They are synthesized by the phenylpropanoid metabolite pathway and have a characteristic structure containing a rearranged C15 skeleton based on 3-phenylchromen-4-one [2,4]. Interestingly, isoflavones possess several biological and physiological properties including antioxidant, anti-inflammatory, and anticancer properties, especially for hormone-dependent breast and prostate cancers, as well prevention of menopausal symptoms, cardiovascular disease, and osteoporosis, and in chemotaxis of nitrogen fixing bacteria [5-9]. Isoflavones are phytoestrogens, which are structurally similar to genuine 17β-estradiol.
and have a weak estrogenic activity; therefore, they can modulate the actions of endogenous estrogens in vertebrates by binding to estrogen receptors (ERs) [10-13].

Chemical synthesis, for example reduction with palladium catalyst on activated carbon (Pd/C) and ammonium formate under N₂ atmosphere [14], is being used to extend the diversity of flavonoids and to improve their biological and physiological properties. However, chemical synthesis generally generates toxic waste products [11]. Microbial transformation offers the advantages of cost-effective, high value organic molecules, and reduces levels of toxic waste products [7-8]. Biotransformation with recombinant microbial enzymes and biomolecular engineering has been widely used, including applications in the production of hormones, antibiotics, and chemicals [8]. Therefore, we tried to produce isoflavone derivatives using microbial transformation.

Polychlorinated biphenyls (PCBs) are an example of environmental contaminants that have multiple effects which disrupt the hormonal system both in animals and humans, and are commonly known as endocrine disrupting chemicals [4,15]. Bioremediation may be an alternative way to restore an environment contaminated with persistent organic pollutants. Biphenyl-degrading bacteria are able to degrade PCBs; biphenyl dioxygenase (BDO) is a key enzyme in the catabolic pathway involved in the degradation of PCBs. It is responsible for the initial dioxygenation step during the metabolism of biphenyl and consists of terminal dioxygenase, ferredoxin, and ferredoxin reductase (Figure 1) [5,8,16,17]. Due to the similarity in the chemical structure of isoflavone and biphenyl, it was assumed that isoflavones are a substrate for BDO.

Alawiyah et al. [16] and Romsaiyud et al. [17] reported that biotransformation of isoflavan-4-ol by BDO resulted in four epoxidation or hydroxylation metabolites, namely CM1, CM2, TM1, and TM2. In this study, we tried to purify CM1 and CM2 and to modify the extraction method to solve the degradation problem during product purification.

2. Methods

Microorganisms, culture conditions and preparation of resting cells. E. coli JM109 (pJHF108) carrying a BDO gene from P. pseudoalcaligenes KF707 was kindly provided by Prof. K. Furukawa from Kyushu University, Japan. Fermentor culture and subculture was grown in LB medium at pH 7.2 with 50 µg/ml of ampicillin and carried out at 37 °C for 12 h in a reciprocal shaker at 200 rpm. The culture was poured to the 5 L fermentor containing 4.5 L of LB medium and 50 µg/ml of ampicillin. The fermentor was constantly aerated and stirred at 250 rpm, 37 °C for 8 h. After 8 h, cultures were harvested and centrifuged at 7,000 rpm, 4 °C, for 10 min. The harvested cells were washed three times with MSB buffer (1 X) and suspended in 1 X MSB buffer. Cell suspensions were used for the biotransformation reaction.

Production of isoflavan-4-ols. Pd/C (50 mg) was added to the isoflavone suspension (100 mg, 0.90 mmol) in anhydrous ethanol (20 ml) together with ammonium
formate (200 mg, 6.53 mmol). The reaction mixture was stirred at room temperature for 24 h under a nitrogen atmosphere. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was dissolved in chloroform (50 mL), washed twice with water and dried over anhydrous MgSO₄. The product was isolated using preparative layer chromatography (PLC) (100% CHCl₃).

Purification of enantiomers of cis-isoflavan-4-ol. To purify each enantiomer of cis-isoflavan-4-ol ((3R, 4R)-cis-isoflavan-4-ol (CE1) and (3S, 4S)-cis-isoflavan-4-ol (CE2)), chiral column chromatography was performed. The column was a Sumi Chiral OA-7000 column (5 µm particle size, 20 mm x 25 cm; Sumika Chemical Analysis Service, Ltd., Osaka, Japan) and the mobile phase consisted of 20 mM phosphate buffer at pH 3.0.

High performance liquid chromatography (HPLC). Biotransformation products of cis-isoflavan-4-ol were analyzed using HPLC Varian Prostar (Walnut Creek, CA, USA) equipped with a Waters Spherisorb® ODS-2 C18 column (5 µm particle size, 4.6 mm x 250 mm) and UV detection was carried out at 270 nm. The flow rate was 18 ml/min and UV detection was carried out at 270 nm.

Liquid chromatography/mass spectrophotometry (LC/MS). LC/MS was carried out by coupling Alliance 2695 (Waters Corporation, Milford, MA, USA) with a Quattro LC triple-quadrupole tandem mass spectrometer (Waters Corporation) in positive electrospray ionization mode. The mobile phase and elution program were the same as analytical HPLC, the flow rate was set at 0.2 ml/min and UV detection was performed with a photodiode array detector 2996 (Waters Corporation); the injection volume was 10 µL. The source temperature, desolvation temperature, cone and capillary voltage, and electron multiplier voltage, were set at 150 °C, 350 °C, 25 V and 3.0 kV, and 700 V, respectively. Nitrogen gas was used as the cone gas and set at 30 l/h and ultra-pure nitrogen gas was used as the desolvation gas and set at 500 l/h.

3. Results and Discussion

The reduction of alkenes, alkynes, nitro groups, nitriles, imines, aromatic groups, etc. is often carried out by hydrogenation reactions using metal catalysts. The metals used in the reaction are often platinum, palladium, and rhodium. Catalytic transfer of hydrogen is a widely accepted alternative method that does not require the use of potentially dangerous hydrogen gas. The reactions are carried out at room temperature and are rapid, often completed in 10 min or less using excess triethylsilane (TES) and 10–20% Pd/C (by weight) in MeOH. Pd/C is a catalyst that accelerates the rate of reactions but is not itself consumed [18,19].

To produce cis-isoflavan-4-ol, the reduction of isolavone was done by chemically using Pd/C and ammonium formate. Two isoflavan-4-ol stereoisomers appeared as the reaction products (Figure 2); they were separated by thin layer chromatography and each stereoisomer fraction was almost pure. No other product apart from the two isoflavan-4-ol stereoisomers was found. Confirmed by NMR data, the stereoisomers were cis-isoflavan-4-ol and trans-isoflavan-4-ol, respectively (Figure 3).

1H NMR spectral data of trans-isoflavan-4-ol: 5.017 (dd, J₁H₂ = 0.3 Hz (unr), J₁H₄ = 5.2 Hz, J₃H₄ = 8 Hz, H-4), 4.400 (dd, J₁H₂ = 11.2 Hz, J₃H₄ = 3.6 Hz, J₄H₄ = 5.2 Hz (unr), H-2β), 4.292 (dd, J₁H₂ = 11.2 Hz, J₃H₄ = 8.4 Hz, J₄H₄ = 0.3 Hz (unr), H-2α), 3.211(dt, J₁H₂ = 8.4 Hz, J₄H₄ = 3.6 Hz, J₄H₄ = 8 Hz, H-3), 3.374 (dt, J₁H₂ = 8 Hz, H-3), 3.000 (dd, J₃H₄ = 5.2 Hz (unr), J₄H₄ = 11.8 Hz, H-3), 2.321 (dd, J₁H₂ = 11.8 Hz, J₃H₄ = 3.6 Hz, J₄H₄ = 1.2 Hz, H-2α), 1.374 (dt, J₁H₂ = 3.6 Hz, J₄H₄ = 11.8 Hz, J₄H₄ = 1.2 Hz, H-3), 1H NMR of cis-isoflavan-4-ol (400MHz, CDCl₃, ppm): 4.852 (br s, J₁H₂ = 12 Hz (unr), J₁H₄ = 0.2 Hz (unr), J₃H₄ = 3.4 Hz (unr), H-4), 4.652 (dd, J₁H₂ = 12Hz, J₃H₄ = 11.8 Hz, J₄H₄ = 0.2 Hz (unr), H-2β), 4.377 (ddd, J₁H₂ = 12 Hz, J₃H₄ = 3.6 Hz, J₄H₄ = 1.2 Hz, H-2α), 2.374 (dt, J₁H₂ = 3.6 Hz, J₄H₄ = 11.8 Hz, J₄H₄ = 1.2 Hz, H-3). trans-isoflavan-4-ol was characterized by a singlet of H-4 at 5.017 ppm, and H-3, H-2α, H-2β were observed at 3.211 ppm, 4.400 ppm, and 4.292 ppm. While cis-isoflavan-4-ol was characterized by a singlet...
of H-4 at 4.852 ppm, and H-3, H-2α, H-2β were observed at 3.374 ppm, 4.377 ppm, and 4.652 ppm, respectively.

Chemical reactions are being used to extend the diversity of flavonoids. In addition to chemical reaction, which generally generates toxic waste products, microbial transformation also could be used. cis-isoflavan-4-ol modification was carried out using the recombinant microbial enzyme BDO from *P. pseudoalcaligenes* expressed in *E. coli*; BDO is able to biotransform biphenyl compounds in the laboratory [8]. Due to the similarity in the chemical structure of isoflavone and biphenyl, it is assumed that isoflavone is a substrate for BDO. Enantiomers of *cis*-isoflavan-4-ol were purified by a preparative HPLC using a chiral column. Each enantiomer was designated as CE1 and CE2 (Figure 4). Recently, the absolute configurations of CE1 and CE2 were determined as (3R, 4R)-*cis*-isoflavan-4-ol and (3S, 4S)-*cis*-isoflavan-4-ol, respectively [9]. The two enantiomers of *cis*-isoflavan-4-ol (CE1 and CE2) were used as substrates for the biotransformation by *E. coli* expressing BDO from *P. pseudoalcaligenes* KF707 and the biotransformation products were called *cis* metabolite 2 (CM2) and *cis* metabolite 1 (CM1), respectively.

![Figure 2. HPLC Chromatogram of cis- and trans-isoflavan-4-ol: (A) Products from Chemical Reduction of Isoflavone, (B) cis-isoflavan-4-ol after Purification with Preparative Thin Layer Chromatography, (C) trans-isoflavan-4-ol after Purification with Preparative Thin Layer Chromatography](image)

![Figure 3. The Structures of cis- and trans-isoflavan-4-ol Based on the NMR Data](image)
According to HPLC analysis, the biotransformation products of cis metabolite (CM1 and CM2) showed different retention times at 11.9 and 14.6 min; however, they had the same absorption peaks at 204, 220, and 275 nm, respectively (Figure 5). Based on the data, it can be said that CM1 and CM2 had the same molecular structure but different conformational isomers. Generally, chromatographic retention-structure relationships supplement the information provided by the UV spectra. Retention can provide information on the size, acidity, or basicity of the modification groups as well as the position of the modification. Modifications such as methylation, dehydroxylation, ribosylation, and phosphorylation will result in a large change in HPLC retention time, but this does not affect the HPLC spectrum. Ribonucleotide-performance liquid chromatography has a high selectivity for isomers; structural isomers having the same modification at different positions can be identified from their spectral similarities and characteristic retention times. Further, it can be used to separate conformational isomers, the α isomer eluting earlier than the β isomer [20].

Kinetics of the biotransformation of (3R, 4R)-cis-isoflavan-4-ol and (3S, 4S)-cis-isoflavan-4-ol was carried out using E. coli expressing BPO from P. pseudoalcaligenes. The biotransformation kinetics data for (3R, 4R)-cis-isoflavan-4-ol (CE1) and (3S, 4S)-cis-isoflavan-4-ol (CE2) showed that the concentration of CM2 increased and until 12 h and almost all of (3R, 4R)-cis-isoflavan-4-ol (CE1) was transformed to CM2 (Figure 6A). In case of (3S, 4S)-cis-isoflavan-4-ol (CE2), maximal activity was obtained within 8 h and only 30% of (3S, 4S)-cis-isoflavan-4-ol (CE2) transformed to CM1 (Figure 6B).

Cis metabolites (CM1 and CM2) were purified from reaction solution by preparative HPLC using C18 column. The fraction containing CM1 and CM2 were extracted with ethyl acetate and evaporated. The residue...
was dissolved in methanol and analyzed by HPLC. Unfortunately, the final fraction contained two other more hydrophobic products (HP1 and HP2). To find differences among original products cis metabolites CM1 and CM2 and the hydrolysis products (HP1 and HP2), they were analyzed with LC/MS. They all had the same \([M-H_2O+H]^+\) at m/z 225 (Figure 7) and also showed the same UV spectra. Therefore, the hydrolysis products (HP1 and HP2) were probably produced from cis metabolites CM1 and CM2 by acidic hydrolysis (ring opening). Han et al. (2005) reported that biotransformation of flavanone by BDO produced an epoxide functional group on the metabolites [6]. In agreement with Dixon et al. (1999) and Alawiyah et al. (2007) (Figure 1), we proposed that cis metabolites (CM1 and CM2) might be epoxide products of (3R, 4R)-cis-isoflavan-4-ol (CE1) and (3S, 4S)-cis-isoflavan-4-ol (CE2), respectively, and HP1 and HP2 are hydroxylated products of CE1 and CE2, respectively [5,16].

Biotransformation of (iso)flavonoid substrates used two different dioxygenase molecules could regioselectively hydroxylate flavonoids to produce different metabolites (i.e., BDO preferentially hydroxylates the flavonoid B-ring while naphthalene dioxygenase (NDO) only attacks the flavonoid A-ring). Biotransformation of B-ring skewed substrates, flavanone and isoflavonol, by BDO from \(P.\) pseudoalcaligenes produced the epoxide products, instead of dihydrodiols [21]. When flavone was biotransformed with \(E.\) coli JM109 strain expressing BDO from \(P.\) pseudoalcaligenes KF707, cis-flavone 2',3'-dihydrodiol was identified from HPLC as the only metabolite produced [6,23]. In addition to flavone biotransformation, biotransformation of isoflavone with BDO, a structural isomer of flavone with the B-ring at C3, also produced the corresponding isoflavone cis-2',3'-dihydrodiol in the same way [22].

To solve the hydroxylated products during evaporation, the extraction solvent and scheme were modified.
Figure 8. The Reaction of Biotransformation of cis-isoflavan-4-ol Using *E. coli* Expressing BDO from *P. pseudoalcaligenes*

Figure 9. Separation of Biotransformation Product from cis Metabolite Through Chloroform Extraction
Chloroform was used as the extraction solvent instead of ethyl acetate. One volume of chloroform was added to the reaction solution and even after five times extraction, a small amount of cis metabolites was still present (Figure 9). Therefore, chloroform extraction should be performed at least five times. The ethyl acetate extraction step for the preparative HPLC fractions was removed and an evaporation step was included to remove acetonitrile from the HPLC fraction and freeze drying step. A hexane extraction step before chloroform extraction was also included.

4. Conclusions

Biotransformation of (3R, 4R)-cis-isoflavan-4-ol and (3S, 4S)-cis-isoflavan-4-ol by BDO from P. pseudoalcaligenes KF707 produced cis metabolites (CM1 and CM2), which were assumed to be epoxide metabolites. The extraction and purification with chloroform instead of ethyl acetate yielded a high amount of the metabolites in the fifth repetition. Furthermore, purification of CM1 and CM2 was performed with a modified procedure and the structure of CM1 and CM2 were determined by NMR.

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