

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 as antioxidant and anticancer. Biotransformation of two enantiomers (CE1 and CE2) of cis-isoflavan-4-ol by *E. coli* JM109 (pJHF108) carrying a biphenyl dioxygenase gene from *P. pseudoalcaligenes* KF707 produced two products and they were designated as CM1 and CM2. They had retention time at 11.9 and 14.6 min, respectively, and same absorption peaks at 204, 220 and 275 nm. CM1 and CM2 had $[M-H_2O+H]^+$ at m/z 225. Based on the molecular mass, hydrolysis products, and previous report, this study proposed that epoxidation occurred on cis-isoflavan-4-ol. Chloroform extraction was done to improve the stability of CM1 and CM2.

Key words : biotransformation, isoflavone, BDO, *P. pseudoalcaligenes*

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, as flavonols, flavones, flavanones, flavan-3-ols (or catechins), isoflavones, and anthocyanidins (Graf *et al.*, 2005). It has characteristic structure and contains a rearranged C15 skeleton based on 3-phenylchromen-4-one (Akashi *et al.*, 1999; Schantz, 2001).

Interestingly, isoflavones possess several biological and physiological properties including antioxidant, anti cancer especially hormone-dependent breast and prostate cancers, and in chemotaxis of nitrogen fixing bacteria (Dixon and Steele, 1999; Seeger *et al.*, 2003; Han *et al.*, 2005; Miyazawa, 2006; Won *et al.*, 2008). Isoflavones are phytoestrogens which structurally similar with genuine 17β - estradiol and have weak estrogenic activities, therefore they can modulate the actions of endogenous estrogens in vertebrates by binding to estrogen receptor (ERs) (Bayer *et al.*, 2001; Hodex, 2002; Erdman, 2005; Zhang, 2007).

To extend the diversity of flavonoids and to improve biological and physiological properties, chemical synthesis is being used, for example by reduction with catalyst palladium on activated carbon (Pd/C) and ammonium formate under N_2 atmosphere (Seunaga *et al.*, 2002). However, chemical synthesis generally generates toxic waste products (Erdman, 2005). Microbial transformation offers the advantages of cost-effective, high value organic molecular, and reduce levels of toxic waste products (Seeger *et al.*, 2003; Miyazawa, 2006). Biotransformation with recombinant microbial enzymes and biomolecular engineering has been widely used, including application for the production of hormones, antibiotics, and chemicals (Seeger *et al.*, 2003). Therefore, we tried to produce isoflavone derivatives using microbial transformation.

Polychlorinated biphenyls (PCBs) is one example of environmental contaminants which have multiple effects to disrupt hormonal system both in animal and human, and is commonly known as endocrine disrupting chemicals (EDCs) (Schantz *et al.*, 2001; Ang *et al.*, 2005). Bioremediation may be an alternative way to restore contaminated environment

from persistent organic pollutants (POPs). Biphenyl-degrading bacteria are one example which able to degrade PCBs. Biphenyl dioxygenase (BDO) is key enzyme of catabolic pathway which is involved in degradation of PCBs. It is responsible for the initial dioxygenation step during metabolism of biphenyl, and consisting of terminal dioxygenase, ferredoxin, and ferredoxin reductase (Figure 1) (Dixon *et al.*, 1999; Seeger *et al.*, 2003; Alawiyah *et al.*, 2007; Romsaiyud *et al.*, 2008). Due to the similarity chemical structure of isoflavone and biphenyl, we assumed that isoflavones are substrate for biphenyl dioxygenase.

Alawiyah *et al.* (2007) and Romsaiyud *et al.* (2007) reported that biotransformation of isoflavan-4-ol by BDO resulted in four epoxidation or hydroxylation metabolites such as CM1, CM2, TM1, and TM2. In this study, we tried to purify CM1 and CM2 and to modify extraction method to solve degradation problem during product purification.

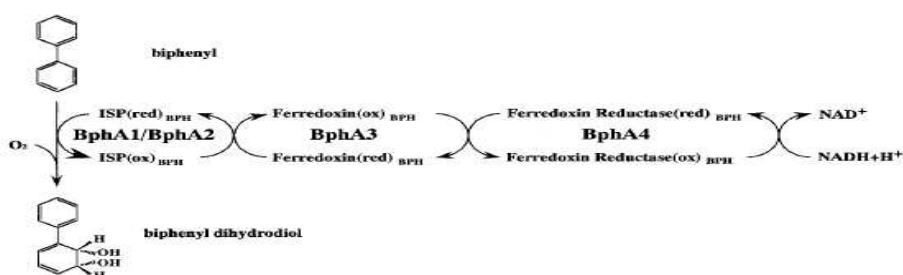


Figure 1. Dioxygenation of biphenyl by BDO *P. pseudoalcaligenes* KF707.

Material and methods

Microorganism, culture condition and preparation of resting cells. *E. coli* JM109 (pJHF108) carrying a BDO gene from *P. pseudoalcaligenes* KF707 was kindly provided by Professor K. Furukawa at Kyushu University, Japan. Fermentor culture, subculture was grown in LB medium pH 7.2 and 50 µg/mL of ampicillin and carried out at 37°C for 12 hrs reciprocal shaker 200 rpm. The culture was poured to the 5 L fermentor containing 4.5 L LB medium and 50 µg/mL of ampicillin. The fermentor was constantly aerated and stirred at 250 rpm, 37 °C for 8 hrs. After 8 hrs, cultures were harvested and centrifuged at 7000 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. Cells suspension was used for the biotransformation reaction.

Production of isoflavan-4-ols. Palladium on activated carbon (50 mg) was added to suspension isoflavone (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 hrs under nitrogen atmosphere. The catalyst was removed by filtration and filtrate was concentrated under reduced pressure. The residue was dissolved in chloroform (50 mL), washed with water two times 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 (CE1 and CE2), chiral column chromatography was carried out. 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 pH 3.0 and acetonitrile (70 : 30, v/v). Flow rate was 18 ml/min and UV detection was done at 270 nm.

Biotransformation kinetics of enantiomers of cis-isoflavan-4-ol by BDO. To study biotransformation kinetics of CE1 and CE2, resting cells of *E. coli* (OD 600 = 10) was mixed with CE1 or CE2 and glucose at final concentration 0.1 mM and 1 M, respectively, and incubated at 37°C with reciprocal shaker 200 rpm and samples were analyzed every 2 hrs during incubation time. To analyze metabolite, the reaction solution was centrifuged and supernatant extracted with three volume ethyl acetate. The ethyl acetate layer was

filtered on sodium sulfate and concentrated using rotary evaporator. The residue was dissolved in methanol and followed by filter using PVDF syringe filters and analyzed by HPLC. In the modified extraction method, culture supernatant was extracted with one volume of chloroform five times.

Purification of CM1 and CM2. To purify CM1 and CM2, HPLC Varian Prostar (Walnut Creek, CA) with a Waters Spherisorb® ODS-2 C18 column (5 µm particle size, 4.6 mm x 250 mm) was used. Flow rate was 18 mL/min and detection was done at 270 nm. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). The elution program was performed: 10% solvent B at 0 min, 40 % solvent B for 20 min, 90% solvent B for 15 min.

High performance liquid chromatography (HPLC). Biotransformation products of *cis*-isoflavan-4-ol was analyzed using HPLC Varian Prostar (Walnut Creek, CA) equipped with a Waters Spherisorb® ODS-2 C18 column (5 µm particle size, 4.6 mm x 250 mm). Flow rate was maintained at 1 mL/min and the detection was done at 270 nm. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and acetonitrile (solvent B). The elution program was as follows: 10% solvent B at 0 min, 40 % solvent B for 15 min, 90% solvent B for 10 min, and by 10 min equilibrium.

Liquid chromatography/ mass spectrophotometry (LC/MS). LC/MS was carried out by coupling Alliance 2695 (Waters Corporation, Miford, MA) with a Quattro LC triplequadruple tandem mass spectrometer) Waters Corporation, Miford, MA) in positive electrospray ionization (ESI+) mode. The mobile phase and elution program were same as analytical HPLC and flow rate was 0.2 mL/min and UV detection was performed with photo diode array detector 2996 (Waters Corporation, Miford, MA). Injection volume was 10 µL. The source temperature, desolvation temperature, cone voltage 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 for cone gas and set at 30 L/h and ultra pure nitrogen gas was used for desolvation gas and set at 500 L/h.

Result and Discussion

Production of isoflavan-4-ols. To produce *cis*-isoflavan-4-ol, reduction of isoflavone was carried out using catalyst palladium on activated carbon (Pd/C) and ammonium formate. Two isoflavan-4-ol stereoisomers appeared as products (Figure 2). Confirmed with NMR data, they were *cis*-isoflavan-4-ol and *trans*-isoflavan-4-ol, respectively (data not shown). They were separated by thin layer chromatography and each stereoisomer fraction was almost pure (Figure 3B and 3C).

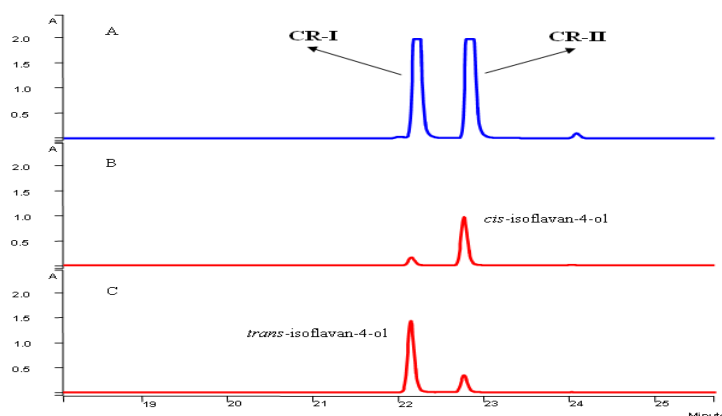


Figure 2. HPLC chromatogram of *cis*- and *trans*-isoflavan-4-ol. (A) Products from chemical reduction of isoflavone, (B) *Cis*-isoflavan-4-ol after purification with preparative TLC, (C) *Trans*-isoflavan-4-ol after purification with preparative TLC.

Purification of enantiomers of *cis*-isoflavan-4-ol. Enantiomers of *cis*-isoflavan-4-ol were purified by a preparative HPLC using chiral column. Each enantiomer was designed as CE1 and CE2 (Figure 3). Recently, the absolute configuration of CE1 and CE2 was determined as (3R, 4R)-*cis*-isoflavan-4-ol and (3S, 4S)-*cis*-isoflavan-4-ol, respectively (Won *et al.*, 2008).

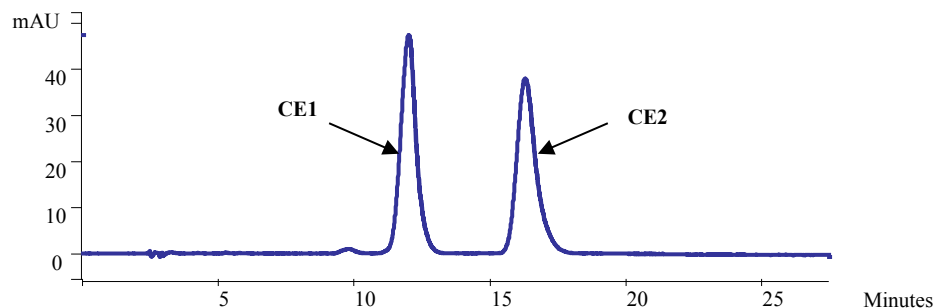


Figure 3. Separation of *cis*-isoflavan-4-ol enantiomers by chiral column chromatography

Biotransformation of CE1 and CE2 by BDO. Two enantiomers of *cis*-isoflavan-4-ol (CE1 and CE2) were used as a substrate for the biotransformation by BDO. Purified CE1 and CE2 were incubated with BDO and the product of CE1 and CE2 were assigned as CM2 and CM1, respectively (Figure 4).

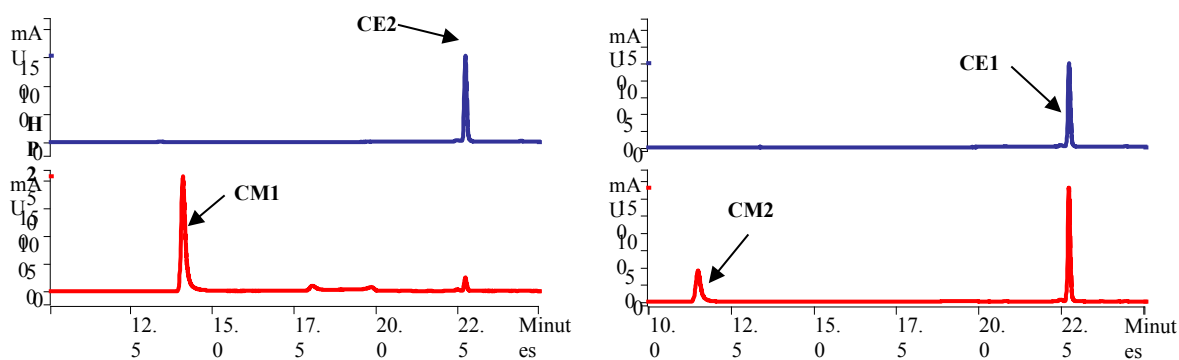
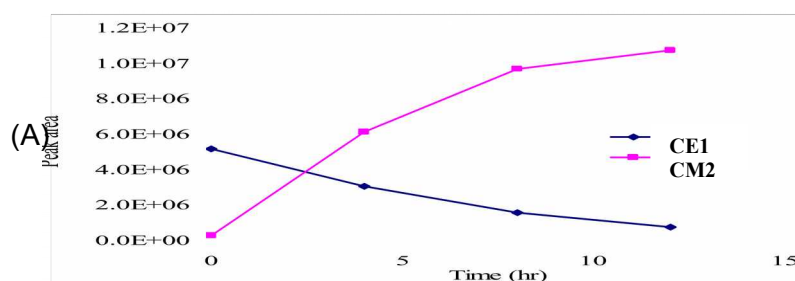


Figure 4. HPLC chromatograms of biotransformation products produced from CE2 (A) and CE1 (B) by BDO. Blue line: 0 hr; Red line: 12 hr.



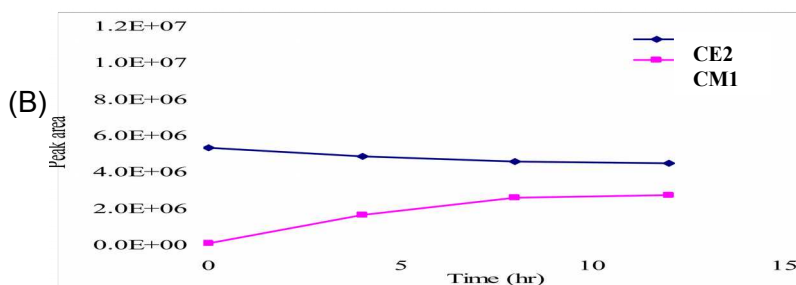


Figure 5. Biotransformation kinetics of CE1 (A) and CE2 (B) by BDO

According to HPLC analysis, CM1 and CM2 showed retention time at 11.9 and 14.6 min and they had the same absorption peaks at 204, 220 and 275 nm, respectively. The biotransformation kinetics was studied for CE1 and CE2, it showed that the concentration of CM2 increased and until 12 hrs and almost all of CE1 was transformed to CM2 (Figure 5A). In case of CE2, maximal activity was obtained within 8 hrs and only 30 % of CE2 transformed to CM1 (Figure 5B).

Purification of CM1 and CM2. 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).

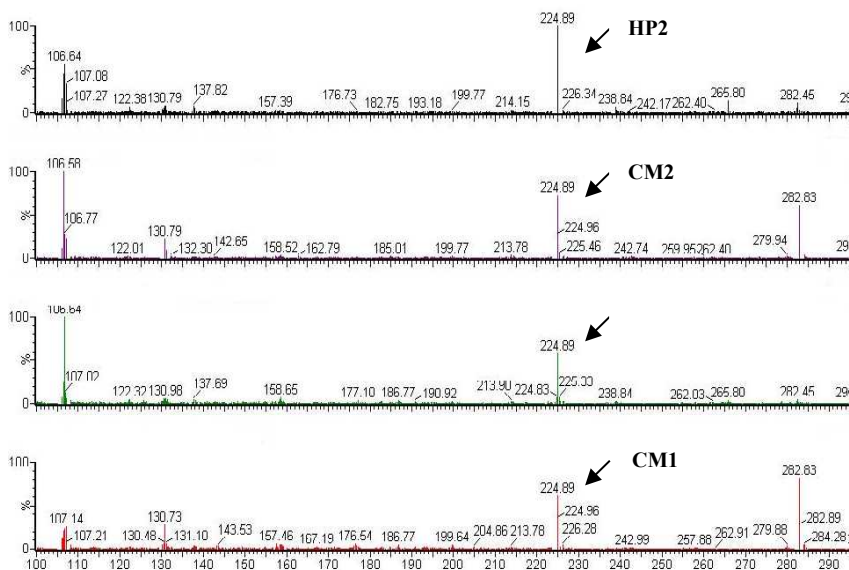


Figure 6. LC/MS spectra of CM1, CM2 and HP2.

To find differences among original products (CM1 and CM2) and hydrolysis products (HP1 and HP2), they were analyzed with LC/MS. All of them have same $[M-H_2O+H]^+$ at m/z 225 (Figure 6) and also showed same UV spectra. We thought that HP1 and HP2 were produced from CM1 and CM2 by acidic hydrolysis (ring opening). Han *et al.* 2005 reported that biotransformation product of flavanone by BDO produced an epoxide functional group on the metabolites. Therefore, we proposed that CM1 and CM2 might be epoxide product of CE1 and CE2, respectively, and HP1 and HP2 are hydroxylated products of CE1 and CE2, respectively.

To solve the hydroxylated products, during evaporation, we tried to modify extraction solvent and scheme. Instead of ethyl acetate, chloroform was used as extraction solvent. One volume of chloroform was added to the reaction solution and even after five times extraction, still a little of CM1 was left (data not shown). Therefore we concluded that chloroform extract should be done at least five times. We removed ethyl acetate extraction step for the preparative HPLC fractions and included evaporation step to remove acetonitrile from HPLC fraction and freeze drying step. We also included hexane extraction step before chloroform extraction.

Conclusion

Cis-isoflavanol was produced from isoflavone by chemical reduction method and each enantiomer (CE1 and CE2) were purified by preparative HPLC. *E. coli* expressing biphenyl dioxygenase transformed CE1 and CE2 to CM2 and CM1, respectively. During ethyl acetate extraction and evaporation step following preparative HPLC, non-target products (HP1 and HP2) were produced. We modified extraction step using chloroform instead of ethyl acetate. Based on the molecular mass, hydrolysis products, and previous result [9], we proposed that CM1 and CM2 are epoxidized product of CE2 and CE1, respectively. Furthermore, purification of CM1 and CM2 will be done by modified procedure and structure of CM1 and CM2 will be determined by NMR.

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