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Production of bioactive hydroxyflavones by using monooxygenase from *Saccharothrix espanaensis*

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ABSTRACT

Biocatalysts are a valuable tool for the structural modification of fine chemicals. Flavonoids possess several biological activities, which are correlated to their antioxidant activity. The numbers of hydroxyl groups in flavonoids are critical for their antioxidant activity. Development of biocatalysts for hydroxylation of flavonoids is challenging because of the difficulty in expressing flavonoid hydroxylase in *Escherichia coli*. In this study, a monooxygenase from *Saccharothrix espanaensis* (Sam5) was used for regioselective hydroxylation of flavonoids. We found that Sam5 hydroxylated isoflavones, flavanones, and flavones but did not produce any detectable hydroxylated product with flavonols. In addition, coexpression of *P450 reductase* with *Sam5* in *E. coli* enhanced hydroxylation by approximately from 34 to 50%, depending on the flavonoid used. The production of two bioactive flavonoids, 8-hydroxyluteolin and 3′-hydroxydaidzein was optimized using this Sam5 system. Approximately 88 mg/L of 8-hydroxyluteolin and 75 mg/L of 3′-hydroxydaidzein were obtained. These results indicate that the Sam5 system could be used for the production of bioactive hydroxylated flavonoids.

1. Introduction

Biocatalysis is a valuable tool for the production of fine chemicals. Biocatalysis and biotransformation will contribute to 30% of all chemical production by the year 2050 (van Beilen et al., 2003). The reactions mediated by biocatalysis confer chemo-, regio-, and enantio-selectivity to chemicals, all of which are attractive advantages in the field of synthetic chemistry. Various enzymes including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases are used as biocatalysts for chemical transformation (Koeller and Wong, 2001).

Many enzymes have been developed from diverse sources for use as biocatalysts. The recent genome projects for various organisms, and the development of improved DNA synthesis procedures and heterologous expression systems have resulted in marked increase in the number of biocatalysts. Oxygenases have been of particular interest because they can be used for industrial synthesis of fine chemicals (Li et al., 2002; van Beilen et al., 2003; Urlacher and Eiben, 2006; Urlacher and Girhard, 2012). Oxygenases that transfer one oxygen atom to an organic substrate are called monooxygenases. Monooxygenases are classified as heme-containing enzymes, which are represented by P450 monooxygenase and non-heme enzymes, including copper-dependent monooxygenases and flavin-dependent monooxygenases. Flavin-dependent monooxygenases are important monooxygenases that catalyzes diverse biochemical reactions, including hydroxylation, epoxidation, halogenation, Baeyer–Villiger oxidations, and sulfoxidation (van Berkel et al., 2006). Flavin-dependent monooxygenases use NAD(P)H and O_2 as cosubstrates. NAD(P)H reduces FAD and the reduced FAD reacts with oxygen, and the activated oxygen is used for further reaction (Ballou et al., 2005).

Flavonoids are secondary plant metabolites synthesized using phenylpropanoid pathway (Winkel-Shirley, 2001). The composition and kind of flavonoids in plants differ among species. Therefore, it is difficult to obtain sufficient quantities of any particular flavonoid for use as a medicinal food or as a precursor for the development of new medicines. As an alternative approach for obtaining bioactive flavonoids, biotransformation through biocatalysis has been used (Lim et al., 2004; Kim et al., 2005a). A simple modification of flavonoids such as quercetin and naringenin confers new biological activities. O-Methylation and O-glycosylation are major modification reactions in biotransformation. 7-O-Methylation of quercetin and naringenin leads to the formation of rhamnetin and sakuranetin, respectively. These 7-O-methylated flavonoids have new biological activities, which the parental compounds did not possess (Kim et al., 2006a,b, 2010). Glycosylation of quercetin confers new biological activity on the

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resulting product. For example, quercetin 7-O-rhamnoside shows antiviral activity, whereas quercetin did not possess this activity (Kim et al., 2012). However, hydroxylation of flavonoids has not been well studied. Two oxygenases from plants, flavonoid 3′,4′-hydroxylase and flavonoid 6-hydroxylase, have been characterized (de Vetten et al., 1999; Latunde-Dada et al., 2001), but they have not been used as biocatalysts due to the difficulty in expressing them in a heterologous system. Development of a flavonoid hydroxylation system using biocatalysis is important because recent studies have shown that hydroxyl flavonoids are better antioxidants than their parental flavonoids (Esaki et al., 1998, 1999). In addition, 3′-hydroxydaidzein suppresses ultraviolet (UV)-B induced skin cancer (Lee et al., 2011) and modulates multidrug resistance (MDR); therefore, it could be used to enhance the effects of cancer chemotherapy (Lo et al., 2012). The hydroxylated (iso)flavonoids in these studies were prepared by fermentation of soy with fungi or were purchased commercially. Production of cheaper and more accessible sources of hydroxyl (iso)flavonoids requires the development of biocatalysis systems.

The metabolical pathways of bacteria can be used for the modification of natural compounds, which can confer new qualities to natural compounds. Bacterial monooxygenases have been used for hydroxylation (Urlacher and Eiben, 2006). In this study, we used a monooxygenase (Sam5) from *Saccharothrix espanaensis* (Berner et al., 2006) to hydroxylate flavonoids. Using Sam5, we generated hydroxyflavones and hydroxyisoflavones. Among several hydroxylated flavonoids, we optimized the synthesis of two bioactive compounds, 8-hydroxyluteolin, which has anti-inflammatory activity (Moroney et al., 1988), and 3′-hydroxydaidzein which suppresses UV-B induced skin cancer (Lee et al., 2011). To our knowledge, this is the first study to show hydroxylation of flavonoid using biocatalysis.

2. Materials and methods

2.1. Cloning and expression of Sam5 and OsP450 reductase

To clone *Sam5* from *S. espanaensis*, genomic DNA of *S. espanaensis* was isolated with a Qiagen DNeasy tissue kit (Qiagen, Gaitherburg, MD). *Sam5* was amplified by polymerase chain reaction (PCR) with Hot Start Taq DNA polymerase (Qiagen) using the genomic DNA of *S. espanaensis* as the template. PCR was carried out under the following conditions: 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1.5 min amplification at 72 °C. The primers were designed based on a published sequence (accession number DQ357071.1). The forward primer was 5′-ATgaattcGATGACCATCACGTCACCTGCG-3′ and the reverse primer was 5′-CATgcggccgcTCAGGTGCCGGGGTTGATC-3′. To facilitate cloning, the restriction sites for *Eco*RI and *Not*I (lowercase letters) were attached to the forward primer and the reverse primer, respectively. The PCR product was ligated into the pGEMT-easy vector (Promega, Madison, WI, USA) and the resulting plasmid was verified by sequencing. Subsequently, *Sam5* was subcloned into the *Eco*RI/*Not*I sites of the pCDFDuet vector (Novagen, USA). The resulting plasmid was named pC-Sam5.

OsP450 reductase (*OsPR*) was cloned from *Oryza sativa* by the reverse transcription polymerase chain reaction (RT-PCR) described by Kim et al. (2009). To remove the membrane-binding domain of OsPR, primers were designed based on the published database sequence (accession number CAE01547.2). The forward primer was 5′-AAgtcgacATGTTCCGATCCGGTGGAGGGGC-3′ and the reverse primer was 5′-AAgcggccgcTCACCATACGTCACGGAGGT-3′, which contained the restriction sites for *Sal*I and *Not*I, respectively. The forward primer started at the 148th nucleotide of OsPR, which inserted an initiation codon “ATG”. PCR was conducted with Hot Start Taq DNA polymerase using rice cDNA as template. The PCR product was digested with *Sal*I and *Not*I and was subcloned into the corresponding sites of pGEX5X-3 (GE Bioscience, USA). The resulting plasmid was named pG-OsPR.

pC-Sam5 alone or both pC-Sam5 and pG-OsPR were used to transform *Escherichia coli* BL21 (DE3).

2.2. Biotransformation and analysis of reaction products

To test Sam5 activity by whole-cell biotransformation, *E. coli* BL21 (DE3) harboring pC-Sam5 and pGEX or pC-Sam5 and pG-OsPR was inoculated in 2 mL of LB-medium supplemented with 50 μg/mL spectinomycin and 50 μL/mL ampicillin and cultured overnight at 37 °C with rotary shaking. Cells (50 μL) were transferred to 2 mL of fresh LB-medium supplemented with 50 μg/mL spectinomycin and 50 μL/mL ampicillin and were cultured at 37 °C with shaking until the absorbance at 600 nm reached 0.6. At this point, IPTG was added to the culture medium at a final concentration of 1 mM, and the cells were cultured for an additional 24 h at 18 °C. The induced cells were collected by centrifugation, washed twice with M9 medium, and resuspended in fresh M9 medium containing 2% glucose, 50 μg/mL spectinomycine and 50 μL/mL ampicillin. The cell density was adjusted such as to give absorbance of 3.0 at 600 nm. Substrates, naringenin, eriodictyol, apigenin, luteolin, kaemperol, quercetin, genestein, and daidzein were added at a final concentration of 100 μM. The mixtures were cultured at 30 °C with shaking for 3 h. Then, 500 μL of culture was extracted twice with the same amount of ethyl acetate and dried with a vacuum drier maintaining 45 °C. The dried reaction product was dissolved in 100 μL of dimethyl sulfoxide (DMSO), 20 μL of which was subjected to Varian high performance liquid chromatography (HPLC) in a system equipped with a photodiode array detector (PDA). The reaction products were separated using a C18 reverse-phase column (Varian, 4.60 × 250 mm, 3.5 μm particle size) as described by Kim et al. (2007).

The effect of cell concentration on the production of 8-hydroxyluteolin was examined by adjusting the cell concentration to OD_{600} = 1, 2, 3, 4, 5, 7, or 10 by using M9 medium containing 2% glucose and antibiotics after induction of the proteins. Then, 100 μM substrate was added, and the reaction mixture was incubated at 30 °C for 6 h. The rate of conversion of the substrate was determined using *E. coli* harboring pC-Sam5 and pG-OsPR at the concentration of OD_{600} = 7 in a 10-mL reaction volume. Then, 100 μM substrate was added at 0, 4, 10, and 24 h. The reaction product (200 μL) was periodically extracted with ethylacetate as described above. The production of 3′-hydroxydaidzein was also carried out under these conditions, which were optimized for the production of 8-hydroxyluteolin. However, for this reaction, 100 μM daidzein was added at 0, 2, 10, and 24 h.

The mean and standard error of the mean were calculated from triplicate experiments. Analysis of variance (ANOVA) was carried out using Tukey’s method, with a significance level set at $P = 0.01$ using 2010 Microsoft Office Excel.

2.3. Determination of the reaction product structures

To determine the structure of the reaction products, proteins from 500 mL of *E. coli* harboring pC-Sam5 and pG-OsPR were induced at 18 °C for 36 h. The cells were harvested and resuspended in 1 L of M9 medium containing 2% glucose, 50 μg/mL spectinomycin, 50 μL/mL ampicillin and 100 μM of substrate. The reaction was carried out using a Biotron fermentor (Korea), which maintained the cultures at 30 °C and 500 rpm for 24 h. The culture was extracted twice with an equal amount of ethyl acetate. The organic layer was evaporated using a rotary evaporator, dissolved in 10 mL methanol, and transferred to 1.5 mL Eppendorf tubes. The samples