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Metabolism of 7-ethoxycoumarin, safrole, flavanone and hydroxyflavanone by cytochrome P450 2A6 variants

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ABSTRACT: CYP 2A6 is a human enzyme that metabolizes many xenobiotics including coumarin, indole, nicotine and carcinogenic nitrosamines. The gene for CYP2A6 is polymorphic. There are few data available to clarify the relationship between P450 genetic variants and the metabolism of materials in food. The CYP 2A6 wild-type protein and 13 mutants (CYP2A6.1, CYP2A6.2, CYP2A6.5, CYP2A6.6, CYP2A6.7, CYP2A6.8, CYP2A6.11, CYP2A6.15, CYP2A6.16, CYP2A6.17, CYP2A6.18, CYP2A6.21, CYP2A6.23 and CYP2A6.25) were co-expressed with NADPH-cytochrome P450 reductase in *E. coli*. The hydroxylase activities toward 7-ethoxycoumarin, coumarin, safrole, flavanone and hydroxyflavanone were examined. Ten types of CYP2A6 variants except for CYP2A6.2, CYP2A6.5 and CYP2A6.6 showed Soret peaks (450 nm) typical of P450 in the reduced CO-difference spectra and had 7-ethoxycoumarin *O*-deethylase activities. CYP2A6.15 and CYP2A6.18 showed higher activities for safrole 1′-hydroxylation than CYP2A6.1. CYP2A6.25 and CYP2A6.7 had lower safrole 1′-hydroxylase activities. CYP2A6.7 had lower flavanone 6- and 2′-hydroxylase activities, whereas CYP2A6.25 had higher 6-hydroxylase activity and lower 2′-hydroxylase activity. Hydroxyflavanone was metabolized by CYP2A6.25, but was not metabolized by wild-type CYP2A6.1. These results indicate that CYP2A6.25 possessed new substrate specificity toward flavonoids. Copyright © 2012 John Wiley & Sons, Ltd.

Key words: HPLC; monooxygenase; polymorphism; flavanone; P450

Introduction

The cytochrome P450 monooxygenases are capable of the metabolism of a number of substrates. Enzymes from the CYP family metabolize toxic natural products present in the diets of many terrestrial vertebrates and insects [1–3]. Besides the degradation of chemicals native to the environment, these enzymes have also been shown to metabolize highly complex procarcinogenic atmospheric pollutants such as PAHs (polycyclic aromatic hydrocarbons) – organic compounds containing two or more fused aromatic or heterocyclic rings [4].

Human CYP 2A6 is a major hepatic isoform specifically involved in the oxidative metabolism of nicotine [5], a major constituent in tobacco smoke [6]. CYP2A6 also metabolizes pharmaceutical agents such as methoxyflurane, halothane,

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losigamone, letrozole, valproic acid, disulfiram and fadrozole and activates some procarcinogens such as 4-methylnitrosoamino-1-(3-pyridyl)-1-butanone and *N*-nitrosodiethylamine [7].

There are genetic polymorphisms present in the *CYP2A6* genes. Some variants have been reported to decrease or abolish enzymatic activity. Alleles possessing a single nucleotide polymorphism (SNP), such as *CYP2A6**2*(L160H) [8], *CYP2A6*6* (R128Q) [9], *CYP2A6**7*(I471T) [10], *CYP2A6*11* (S224P) [11] and *CYP2A6*17*(V365M) [12], have been reported. These gene products showed decreased enzymatic activities *in vitro* and *in vivo*. CYP2A6.15, CYP2A6.16 and CYP2A6.21 exhibited coumarin 7-hydroxylation activities [13]. CYP2A6.25 showed a decrease of nicotine C-oxidation [14]. CYP2A6.5 expressed in yeast was a very unstable enzyme [15]. CYP2A6.18 showed coumarin 7-hydroxylation and 5-fluorouracil formation [16]. *CYP2A6*8* is unlikely to affect catalytic activity *in vivo* [17]. *CYP2A6*23* allele impairs enzyme function *in vitro* and *in vivo* and trends toward an association with a lower risk of smoking [18].

The functional consequences of these mutations on the enzymatic catalytic activity were mainly examined for coumarin 7-hydroxylase activities and nicotine C-oxidation [12–14,19]. There are few reports that clarify the relationship between the other possible substrates and CYP2A6 variants.

At present, there is an increasing market for food products with perceived and real health benefits. This development, combined with the consumers' perception that 'natural equals safe', results in a tendency for the increased use of botanical products as bioactive ingredients in functional foods, as food supplements and as herbal teas and food flavors. However, in spite of a long history of use, botanical or herb-based preparations may contain individual ingredients known to be toxic and even genotoxic and carcinogenic. The present article focuses on safrole and flavonoids in food.

Safrole (1-allyl-3,4-methylenedioxybenzene) is a natural constituent of a number of spices such as nutmeg, mace cinnamon, anise, black pepper and sweet basil. Safrole is also present in cola drinks. Safrole is the main component of *Piper betel* inflorescence [20] that is frequently included in the Taiwanese betel quid for its aromatic flavor. Safrole is an alkenylbenzene that is recognized to be a genotoxic carcinogenic agent in rodents [21–23]. Its hepatocarcinogenicity was reported to correlate closely with its DNA adduct formation in the liver [24]. The cytochrome P450s (CYP1A2, 2A6, 2C9, 2C19, 2D6 and 2E1) mediate the bioactivation of safrole to its proximate carcinogenic metabolite, 1′-hydroxysafrole [25]. Polymorphism in P450s, leading to poor metabolizer phenotypes, may reduce the relative risk of the harmful effects of safrole.

Flavonoids are a diverse group of natural products consisting of more than 500 compounds. Many beneficial pharmacological properties have been attributed to flavonoids including antioxidant, anti-inflammatory, anticarcinogenic and chemopreventive activities [26,27]. Many flavonoids are metabolized by mammalian hepatic microsomes [28–31]. Flavanone, a flavonoid, has important antioxidant activities [32–35]. Flavanone is the precursor to all flavonoid structures. Other flavonoids may have mutagenic and/or prooxidant effects [31,36] and depending on the P450 polymorphism, a beneficial effect may be shifted to an antioxidant or mutagenic effect. CYP1A1, CYP1A2 and CYP2B6 convert flavanone to flavone and 2,3-*trans*-flavanonol [37].

In order to reveal the effect of SNPs within human *CYP2A6* on the metabolism of 7-ethoxycoumarin, safrole, flavanone and hydroxyflavanone, this study used recombinant CYP2A6 enzyme variants overexpressed in *Escherichia coli*.

Materials and Methods

Materials

Isopropyl-thio-β-D-thiogalactopyranoside, methanol, flavanone, safrole, 7-ethoxycoumarin, coumarin, 7-hydroxycoumarin and ethyl acetate were obtained from Wako Pure Chemical Industries (Osaka, Japan). All chemicals were of the purest grade commercially available. The QuickChange site-directed mutagenesis system was from Stratagene (LaJolla, CA). 6-Hydroxyflavanone and 2′-hydroxyflavanone were from Extrasynthese (Genay Cedex, France) and 4′-hydroxyflavanone and 7-hydroxyflavanone were from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 1′-Hydroxysafrole was a gift from Dr Kunio Issiki (Bioresource Laboratories, Mercian Corporation, Japan).

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Construction of CYP2A6 gene expression vectors

Full-length cDNA fragments of the CYP2A6 gene were amplified by polymerase chain reaction methods from a human liver cDNA library by using gene-specific primers. To generate protein in an *E. coli* system, the *N*-terminal transmembrane sequences were deleted and several amino acid residue substitutions (^{24}WQQRKSK to ^{24}AKKTSSK) were made in the fragments. The amplified fragment was inserted into the *Nde*I and *Sal*I sites of the pT7 blue vector, designated as the pT7-CYP2A6 vector (Takara Bio Inc., Otsu, Japan).

Site-directed mutagenesis on CYP2A6 cDNA was performed using the QuickChange II site-directed mutagenesis system (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The DNA template was a pT7-CYP2A6 vector. Mutagenesis primers that were designed for generating mutant alleles of *CYP2A6*2*(L160H), *CYP2A6*5* (G479V), *CYP2A6*6*(R128Q), *CYP2A6*7*(I471T), *CYP2A6**8(R485L), *CYP2A6*11*(S224P), *CYP2A6*15* (K194E), *CYP2A6*16*(R203S), *CYP2A6*17*(V365M), *CYP2A6*18*(V392F), *CYP2A6*21*(K476R), *CYP2A6*23* (R203C) and *CYP2A6*25*(F118L) are listed in Table 1.

The substitutions were confirmed by DNA sequence analysis.

All plasmids including CYP2A6 variants were cut with *Nde*I and *Sal*I, and the fragment was subcloned into the *Nde*I and *Sal*I sites of the pCW vector containing the human NADPH-P450 reductase cDNA. To verify that the correct inserts were ligated into the plasmids, the inserts were excised and electrophoresed to confirm that they were the correct size.

P450 production and culture conditions

Escherichia coli DH5*a* cells were transformed with the pCW vector containing a P450 insert. The transformed cells were preincubated in Luria broth (LB) containing 100 μg/ml ampicillin at 37 °C for 1 day, and 3 ml of this culture was then added to 300 ml of LB containing 100 μg/ml ampicillin and incubated at 37 °C to an OD_{600} of 0.2–0.3. After the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) and 5-aminolevulinic acid (0.5 mM), cultures were grown at 25 °C for 40–48 h. The cells were centrifuged at $8000 \times g$ for 10 min, suspended in 4 ml of 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, and stored at –80 °C. Membrane fractions were prepared as described previously [38].

The P450 content of the cells was determined by CO-difference spectroscopy as described previously [39] using a dual-beam spectrophotometer (U-3000 T, Hitachi, Tokyo, Japan). Duplicate matched cuvettes of 2 ml of *E. coli* cell suspensions in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol were used to record a baseline spectrum at 400–500 nm. After adding a few grains of sodium dithionite crystals to two cuvettes, carbon monoxide was bubbled through

Table 1. Specifically designed oligonucleotide primers that have been used in the generation of *CYP2A6*2*, *CYP2A6*5*, *CYP2A6*6*, *CYP2A6*7*, *CYP2A6*8*, *CYP2A6*11*, *CYP2A6*15*, *CYP2A6*16*, *CYP2A6*17*, *CYP2A6*18*, *CYP2A6*21*, *CYP2A6*23* and *CYP2A6*25* mutant cDNAs

Primer	Primer sequence
CYP2A6*2	5′CATCGACGCCC**A**CCGGGGCACTG-3′
CYP2A6*5	5′-CCCCAAACAC**G**T**T**GG**T**CTTTGCCACGATCC-3′
CYP2A6*6	5′-GCCAAGCAGCTCC**A**G**C**GCTTCTCCATC-3′
CYP2A6*7	5′-CCTCCCAGTCACC**T**AAGGACA**C**TGACGTGTCCC-3′
CYP2A6*8	5′-CTTTGCCACGATCCCA**C**TAAA**C**TACACCATGAGCT-3′
CYP2A6*11	5′-CAGCTCTATGAGATGTT**C**CCTTCGGTGATGAAACACC-3′
CYP2A6*15	5′-TCTTTGGGGACCGCTTT**G**ACTAT**G**AGGACAAAGAGT-3′
CYP2A6*16	5′-AGTTCCTGTCACTGTT**G**AGCATG**A**TGCTAGGAATC-3′
CYP2A6*17	5′-ATCCAAAGATTTGGAG**A**CA**A**TGATCCCCATGAGTTTGG-3′
CYP2A6*18	5′-TAAGGGCACCGAAGTGTT**T**CCCTATGCTGGG-3′
CYP2A6*21	5′-ATTGACGTGTCCCCCA**G**A**C**ACGTGGGCTTTG-3′
CYP2A6*23	5′-AGTTCCTGTCACTGTTG**T**GCATGATGCTAGGAATC-3′
CYP2A6*25	5′-TCAAAGGCTATGGCGTGGTA**C**TCAGCAACGGGG-3′

Sequences shown are those of the forward primer, the reverse primers contained sequences opposite to those of the forward primer and annealed to the same sequences on opposite strands of the plasmid template. Nucleotides that were changed to make the desired mutation are shown in bold and underlined.

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one cuvette. The resulting trace gave the spectral difference between the dithionite-reduced CO-bound P450 and dithionite-reduced P450. The P450 content was expressed using an extinction coefficient of 91 mM^{-1} cm^{-1}. NADPH-cytochrome c reduction activity was determined as described previously [40].

Assays for 7-ethoxycoumarin O-deethylase and coumarin 7-hydroxylase activity

A total of 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol (total reaction volume, 0.5 ml); the reaction was started by the addition of 7-ethoxycoumarin or coumarin (final concentration, 335 μM). The solution was then shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of 1 ml of ethyl acetate, vortexed for 5 min and centrifuged at 1000 × *g* for 8 min. The upper phase was extracted with 1 ml of ethyl acetate three times, and the resulting solution was air-dried in the dark. Then, 50 μl of methanol or dimethyl sulfoxide was added to reconstitute the pellet, followed by the addition of 2.5 ml of 0.01 M NaOH/0.1 M NaCl. The amounts of de-ethylated products were quantified by using standard solutions of 7-hydroxycoumarin. The concentrations of 7-hydroxycoumarin were determined fluorometrically (excitation maximum at 368 and emission at 530 nm) with a spectrofluorometer (F-2500, Hitachi, Tokyo, Japan) at 25 °C as described previously [41,42]. Since 7-hydroxycoumarin is photosensitive, the working substrate and standard solutions were prepared daily from stock solutions. Each sample was measured in triplicate. Hydroxyl coumarin production of CYP2A6.1 (wild type) was linear with time for 60 min (data not shown). The K_m value of CYP2A6.1 for 7-ethoxycoumarin deethylase activity was 33.5 μM (data not shown). So a concentration of ten times of K_m was used. The assay was done at 335 μM 7-ethoxycoumarin for 30 min.

Assays for safrole, flavanone, and hydroxyflavanone hydroxylase activities, and HPLC analysis

A total of 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol and 1 mM NADPH (total reaction volume, 0.5 ml). The reaction was started by the addition of hydroxyflavanone, safrole or flavanone (final concentration, 335 μM). The incubation mixture was shaken at 37 °C for 30 min in the dark. Stock solutions of substrates were prepared in methanol. Reactions were stopped by the addition of 1 ml of ethyl acetate, vortexed for 5 min and centrifuged at 1000 × *g* for 8 min. The upper phase was extracted with 1 ml of ethyl acetate three times, and the resulting solution was air-dried. The residual material was reconstituted in 50 μl of 50% methanol and 10 μl aliquots were injected into a high-performance liquid chromatography (HPLC) system (LaChrom, Hitachi, Tokyo, Japan) equipped with an L-7100 pump, an L-7300 injector and an L-7400 UV absorbance detector. The metabolites were separated at 40 °C on a TSK-gel Super ODS column (4 μm, 4.6 × 100 mm, Tosoh, Tokyo, Japan) using 40% methanol at a flow rate of 1.2 ml/min. Metabolite peaks were monitored at 210 nm. Each value is the mean of triplicate measurements. Testosterone (100 μM) was added as an internal control after the reaction was stopped by adding ethyl acetate. Statistical analysis was performed by Tukey-Kramer method and Scheffe's *F*-test.

Results

Expression of CYP2A6 variants in E. coli

Fourteen expression vectors were constructed encoding *CYP2A6*1* (wild type), *CYP2A6**2(L160H), *CYP2A6*5*(G479V), *CYP2A6*6*(R128Q), *CYP2A6*7* (I471T), *CYP2A6*8*(R485L), *CYP2A6*11*(S224P), *CYP2A6*15* (K194E), *CYP2A6*16*(R203S), *CYP2A6*17* (V365M), *CYP2A6*18*(V392F), *CYP2A6*21*(K476R), *CYP2A6*23*(R203C) and *CYP2A6*25*(F118L). *Escherichia coli* DH5*a* cells were transformed with the vector constructs, cultured, collected, and the amount of P450 was measured with a spectrophotometer. Whereas cells transformed with empty pCW vector lacked a peak at 450 nm, the reduced CO-difference spectra of the 10 allelic variants showed Soret peaks (450 nm) typical of P450s. CYP2A6.2, CYP2A6.5 and CYP2A6.6 lacked a peak at 450 nm (data not shown). Membrane fractions were isolated and the NADPH-P450 reductase activities were measured. NADPH-P450 reductase

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activities of CYP2A6.1 and CYP2A6.25 were 5.14 and 5.41 nmol/min/mg protein, respectively. The expression levels of CYP2A6.1 and CYP2A6.25 were 135 and 124 pmol/mg total proteins, respectively.

Deethylation of 7-ethoxycoumarin and coumarin 7-hydroxylation

The study first tested if each CYP2A6 variant could *O*-deethylate the drug 7-ethoxycoumarin. 7-Ethoxycoumarin *O*-deethylase activities are shown in Figure 1. Activities of CYP2A6.1, CYP2A6.7, CYP2A6.8, CYP2A6.11, CYP2A6.15, CYP2A6.16, CYP2A6.17, CYP2A6.18, CYP2A6.21, CYP2A6.23 and CYP2A6.25 were 0.34 ± 0.01, 0.25 ± 0.00, 0.30 ± 0.01, 0.35 ± 0.02, 0.45 ± 0.02, 0.37 ± 0.01, 0.35 ± 0.03, 0.44 ± 0.02, 0.29 ± 0.01, 0.31 ± 0.01 and 0.24 ± 0.01 nmol/min/nmol of P450 (mean ± SD), respectively. Data are mean ± SD of three independent experiments. CYP2A6.15 and CYP2A6.18 were 1.3 fold more active than CYP2A6.1. The differences were statistically significant ($p < 0.05$). CYP2A6.7 and CYP2A6.25 had 0.72- and 0.69-fold lower activities than CYP2A6.1, respectively. The differences were statistically significant ($p < 0.01$). CYP2A6.8, CYP2A6.11, CYP2A6.16, CYP2A6.17, CYP2A6.21 and CYP2A6.23 had similar activities to CYP2A6.1. Thus, four CYP2A6 variants (CYP2A6.7, CYP2A6.15, CYP2A6.18 and CYP2A6.25) were used to test for coumarin 7-hydroxylation.

Coumarin 7-hydroxylase activities, which are widely used activity to CYP2A6, were examined using four CYP2A6 variants and compared with wild type. Coumarin 7-hydroxylase activities are shown in Figure 2. The hydroxylase activities of CYP2A6.1, CYP2A6.7, CYP2A6.15, CYP2A6.18 and CYP2A6.25 were 0.39 ± 0.02, 0.12 ± 0.03, 0.43 ± 0.01, 0.34 ± 0.01 and 0.16 ± 0.01 nmol/min/nmol P450 (mean ± SD), respectively. Data are mean ± SD of three independent experiments. These activities were low, compared with the values reported previously [12,16]. CYP2A6.15 had 1.11-fold higher activities than CYP2A6.1, respectively, whereas CYP2A6.7, CYP2A6.18 and CYP2A6.25 were 0.31- 0.89- and 0.41-fold less active than CYP2A6.1, respectively.

The differences in the activities of the mutants (CYP2A6.7 and CYP2A6.25) and wild type were statistically significant ($p < 0.01$). The trends in coumarin 7-hydroxylase activities of the CYP2A6 variants showed similar activity patterns to those observed for 7-ethoxycoumarin *O*-deethylase.

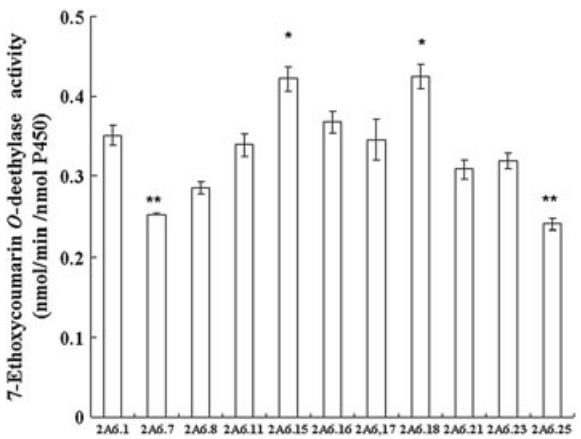

Figure 1. 7-Ethoxycoumarin *O*-deethylase activities of wild type and mutants of CYP2A6. Control, cells were transformed with empty pCW vector. A total of 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol; the reaction was started by the addition of 7-ethoxycoumarin (final concentration, 335 μM). The solution was shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of ethyl acetate. The extracted solution was air-dried in the dark. The amounts of de-ethylated products were quantified by using standard solutions of 7-hydroxycoumarin. SD is represented by vertical bar. $^{**}p < 0.01$,$^{*}p < 0.05$

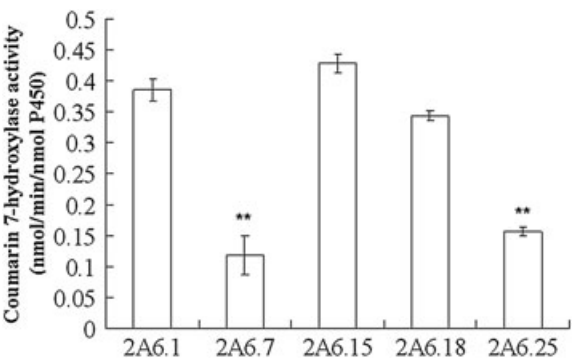

Figure 2. Coumarin 7-hydroxylase activities of wild type and mutants of CYP2A6. Control, cells were transformed with empty pCW vector. A total of 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol; the reaction was started by the addition of coumarin (final concentration, 335 μM). The solution was shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of ethyl acetate. The extracted solution was air-dried in the dark. The amounts of de-ethylated products were quantified by using standard solutions of 7-hydroxycoumarin. SD is represented by a vertical bar. $^{**}p < 0.01$

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Safrole hydroxylation

Safrole hydroxylase activities were examined by HPLC using four CYP2A6 variants and compared with wild type. One product was obtained with a retention time of 3.4 min. The hydroxylase activities are shown in Figure 3. To identify the hydroxylated safrole, metabolite standards of safrole were used. The main hydroxylated product was 1′-hydroxy safrole (data not shown). The hydroxylase activities of CYP2A6.1, CYP2A6.7, CYP2A6.15, CYP2A6.18 and CYP2A6.25 were 1.02 ± 0.08, 0.49 ± 0.01, 1.79 ± 0.01, 1.31 ± 0.18, and 0.73 ± 0.04 nmol/min/nmol P450 (mean ± SD), respectively. Data are mean ± SD of three independent experiments. CYP2A6.15 and CYP2A6.18 had 1.75- and 1.28-fold higher activities than CYP2A6.1, respectively, whereas CYP2A6.7 and CYP2A6.25 were 0.48- and 0.72-fold less active than CYP2A6.1, respectively. The differences in the activities of the mutants (CYP2A6.7 and CYP2A6.15) and wild type were statistically significant ($p < 0.01$, $p < 0.05$). The trends in safrole hydroxylase activities of the CYP2A6 variants showed similar activity patterns to those observed for coumarin 7-hydroxylase.

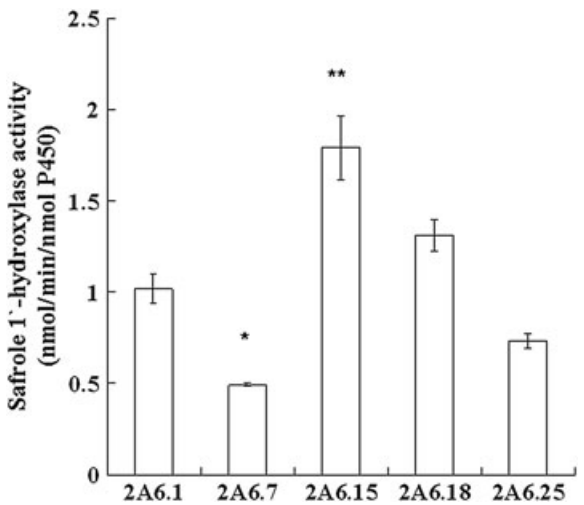

Figure 3. Safrole hydroxylase activities of wild type and mutants of CYP2A6. A total of 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol; the reaction was started by the addition of substrate (final concentration, 335 μM). The solution was shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of ethyl acetate. The extracted solution was air-dried. The residual material was reconstituted in 50 μl of 50% methanol and 10 μl aliquots were injected into an HPLC system. The metabolites were separated at 40 °C on a TSK-gel Super ODS column using 40% aqueous methanol at a flow rate of 1.2 ml/min. Metabolite peaks were monitored at 210 nm. SD is represented by a vertical bar. $^{**}p < 0.01$,$^{*}p < 0.05$

Flavanone hydroxylation

Flavanone was recently reported to be metabolized by mammalian hepatic P450s [43]. The study sought to determine whether CYP2A6 variant enzymes would hydroxylate flavanone. CYP2A6 variants were incubated separately with flavanone and two products were obtained with retention times of 12.3 and 17.7 min (Figure 4). These peaks were identified by comparing with the authentic standards as 6-hydroxyflavanone and 2′-hydroxyflavanone at 12.3 min and 17.7 min, respectively (Figure 4E). These peaks were not observed in the control (Figure 4A and 4B). These results suggest that flavanone is metabolized by CYP2A6 variants. As shown in Figure 5, the 6-hydroxylase activities of CYP2A6.1, CYP2A6.7, CYP2A6.15, CYP2A6.18 and CYP2A6.25 were 0.25 ± 0.00, 0.15 ± 0.01, 0.31 ± 0.02, 0.53 ± 0.04 and 1.56 ± 0.05 nmol/min/nmol P450 (mean ± SD), respectively. Data are mean ± SD of three independent experiments, respectively. CYP2A6.15, CYP2A6.18 and CYP2A6.25 had 1.24-, 2.12- and 6.24-fold higher activities than CYP2A6.1, respectively, whereas CYP2A6.7 was 0.6-fold less active than CYP2A6.1. The differences in the activities of the mutants (CYP2A6.18 and CYP2A6.25) and wild type were statistically significant ($p < 0.01$). The 2′-hydroxylation activities of CYP2A6.1, CYP2A6.7, CYP2A6.15, CYP2A6.18 and CYP2A6.25 were 0.32 ± 0.02, 0.10 ± 0.01, 0.64 ± 0.04, 0.54 ± 0.03 and 0.14 ± 0.01 nmol/min/nmol P450 (mean ± SD), respectively. Data are mean ± SD of three independent experiments. CYP2A6.15 and CYP2A6.18 were 2.0- and 1.69-fold more active than CYP2A6.1, respectively, whereas CYP2A6.7 and CYP2A6.25 were 0.33- and 0.43-fold less active than CYP2A6.1, respectively. The differences in the activities of the mutants and wild type were statistically significant ($p < 0.01$).

The 2′-hydroxylation activities of the two variants were lower than CYP2A6.1, similar to the other substrates, whereas the 6-hydroxylation

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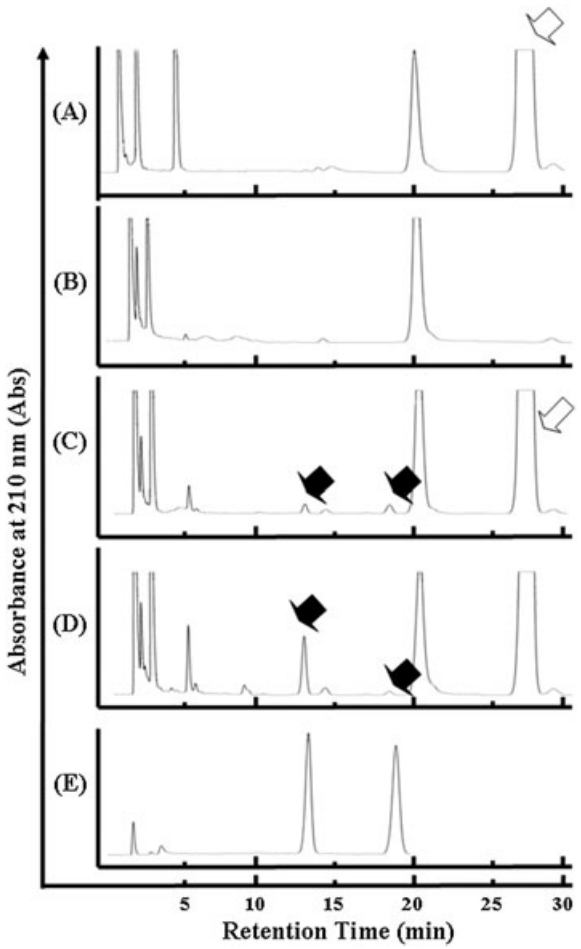

Figure 4. HPLC analysis of flavanone metabolites produced by the action of P450s. Open arrows, substrates; filled arrows, products. (A) Inoculate lacking P450, (B) Inoculate lacking flavanone, (C) Inoculate containing both flavanone and wild type *CYP2A6*1*. (D) Inoculate containing both flavanone and *CYP2A6*25*. (E) 6-hydroxyflavanone and 2′-hydroxyflavanone. The retention times for 6-hydroxyflavanone and 2′-hydroxyflavanone are at 12.3 min and 17.7 min, respectively. Fifty pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol; the reaction was started by the addition of substrate. The incubate was shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of ethyl acetate. The extracted solution was air-dried. The residual material was reconstituted in 50 µl of 50% methanol and 10 µl aliquots were injected into an HPLC system. The metabolites were separated at 40 °C on a TSK-gel Super ODS column using 40% aqueous methanol at a flow rate of 1.2 ml/min. Metabolite peaks were monitored at 210 nm

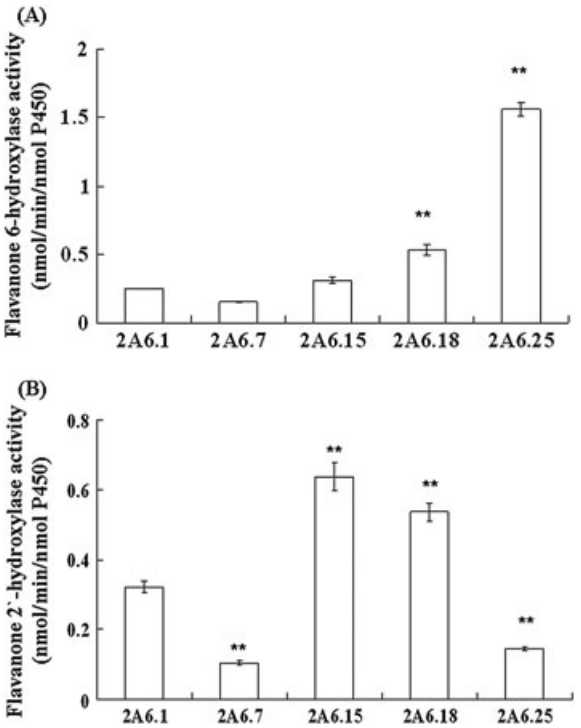

Figure 5. Flavanone hydroxylase activities of wild type and mutants of CYP2A6 (A) 6-hydroxylation (B) 2′-hydroxylation. SD is represented by a vertical bar. $^{**}p < 0.01$

activity of CYP2A6.25 was higher than CYP2A6.1. CYP2A6.25 demonstrated a new substrate specificity toward flavanone. This is the first report to clarify the effect of an amino acid substitution on flavanone hydroxylation.

Hydroxylation of hydroxyflavanones

Next, the metabolism of 4′-hydroxyflavanone by CYP2A6.1 and CYP2A6.25 was investigated. HPLC analysis indicated that the metabolite produced from 4′-hydroxyflavanone by CYP2A6.1 was eluted at 12.4 min, whereas the two metabolites produced by CYP2A6.25 were detected at 7.5 and 12.4 min (Figure 6). Attempts were made to clarify the structure of the metabolites formed using MS/MS, but they were not successful. No product was observed in the reconstituted system containing 2′-hydroxyflavanone, 6-hydroxyflavanone or 7-hydroxyflavanone as a substrate. These results suggest that hydroxyflavanone is metabolized by CYP2A6.1 and CYP2A6.25 demonstrated a new substrate specificity to 4′-hydroxyflavanone. This is the first report to clarify the effect of an amino acid substitution on 4′-hydroxyflavanone hydroxylation.

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Discussion

CYP 2A6 plays a crucial role in the metabolism of many therapeutic drugs as well as metabolic activation of procarcinogens [44–46]. Genetic polymorphism of CYP2A6 is believed to the major cause of individual variation in enzymatic activities for various CYP2A6 substrates and, thus, it is critical to characterize the enzymatic properties caused by the allelic polymorphism [9].

CYP2A6.2 expressed in HepG2 cells showed no activity toward coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation [8]. CYP2A6.2 was not expressed as the active form in *E. coli*. CYP2A6.2 expressed in *E. coli* may be unstable.

*CYP2A6*5* possesses SNPs that cause the amino acid change G479V. When expressed in yeast [15], this substitution resulted in a very unstable enzyme. The *CYP2A6*6* variant contained the R128Q substitution. When expressed in insect cells, the reduced carbon oxide-bound Soret peak was lost [9]. These two substitutions may disorder the holoprotein structure. The structure of CYP2A6 bound with substrate and inhibitor was determined by x-ray crystallography. The CYP2A6 structure shows an enzyme that is well adapted for the oxidation of small planar substrates, such as coumarin, that fit within the narrow, hydrophobic active site cavity of the enzyme [47].

*CYP2A6*7* possesses SNPs that cause I471 to be changed to T. The amino acid change of I471T is known to decrease the enzymatic activities (coumarin 7-hydroxylation and nicotine C-oxidation) *in vitro* and *in vivo* [10,17]. In this study, CYP2A6.7 decreased 7-ethoxycoumarin *O*-deethylation and flavanone and safrole hydroxylation (Figures 2, 3 and 5). The isoleucine at residue 471 is located within the β4(1) strand and near the SRS (substrate recognition site) 6. The amino acid change may affect the folding of SRS 6.

CYP2A6.15 and CYP2A6.18 showed higher activities of 7-ethoxycoumarin deethylation and safrole and flavanone hydroxylation than CYP2A6.1 (Figures 2, 3 and 5). For coumarin 7-hydroxylation, CYP2A6.15 exhibited a higher V_{max} than the wild type [13]. The single point mutation that causes the K194E substitution in variant *CYP2A6*15* is located not within any particular SRS site but adjacent to helix F, which partially embraces SRS-2. Thus, the K194E change may affect the substrate recognition indirectly. Although the tyrosine at residue 392 is located within the β1(3) strand (48) and not in the SRS site, the amino acid change may affect substrate recognition, affinity toward substrate, or turn-over number.

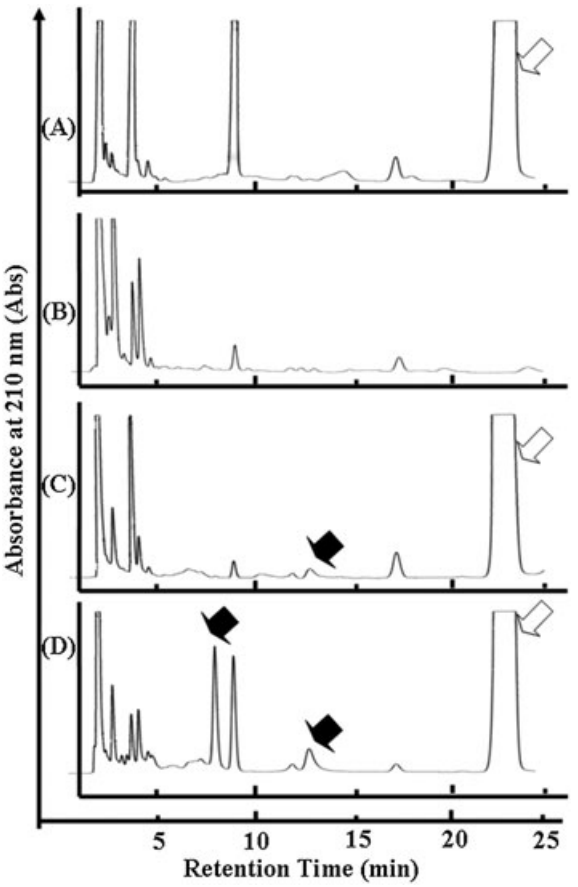

Figure 6. HPLC analysis of the formed metabolites from 4′-hydroxyflavanone by CYP2A6. Open arrows, substrates; filled arrows, products. (A) Inoculate lacking P450, (B) Inoculate lacking hydroxyflavanone, (C) Inoculate containing both hydroxy flavanone and CYP2A6.1. (D) Inoculate containing both hydroxyflavanone and CYP2A6.25. 50 pmol of P450 was added to 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol; the reaction was started by the addition of substrate. The incubate was shaken at 37 °C for 30 min in the dark. Reactions were stopped by the addition of ethyl acetate. The extracted solution was air-dried. The residual material was reconstituted in 50 µl of 50% methanol and 10 µl aliquots were injected into an HPLC system. The metabolites were separated at 40 °C on a TSK-gel Super ODS column using 40% aqueous methanol at a flow rate of 1.2 ml/min. Metabolite peaks were monitored at 210 nm. The metabolite produced from 4′-hydroxyflavanone by CYP2A6.1 was eluted at 12.4 min (C) whereas the two metabolites produced by CYP2A6.25 were detected at 7.5 and 12.4 min (D)

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*CYP2A6*25* exhibits a single amino acid mutation, F118L, which is located in helix B within the highly conserved SRS-1 region of the CYP2A family (13) and close to the coumarin binding site in the x-ray structure [47]. Mwenifumbo *et al*. indicated that CYP2A6.25 showed a lower nicotine C-oxidase activity than CYP2A6.1 [14]. In this study, CYP2A6.25 had lower safrole hydroxylase activity (Figure 3). CYP2A6.25 had lower flavanone 2′-hydroxylation activity, but had higher 6-hydroxylase activity (Figures 4 and 5). Furthermore, a new metabolite of 4′-hydroxyflavanone was detected after incubation with CYP2A6.25 (Figure 6). Obviously, the F118L mutation affects substrate recognition, especially for flavonoids.

Some flavonoids have mutagenic effects and can also interfere with essential biochemical pathways [31,36]. Mutagenicity of flavonoids seems to depend on the number and position of hydroxyl groups in the flavanone. The hydroxylation reactions mediated by CYPs might increase the genotoxicity of the resulting products. Many CYP variants, which are related to genetic polymorphisms, cause reductions in enzymatic activity or result in a complete loss of catalytic activity [48]. There are few reports that show more than two-fold increases in enzymatic activity of CYP mutants. CYP2A6.25 showed an increase in flavanone hydroxylase activity and a new substrate specificity toward hydroxyflavanone, hence F118L substitution may change the genotoxicity of flavonoids.

In conclusion, the CYP 2A6 wild-type protein and 13 mutants were co-expressed with NADPH-cytochrome P450 reductase in *E. coli*. The hydroxylase activities toward 7-ethoxycoumarin, safrole, flavanone and hydroxyflavanone were examined. Ten types of CYP2A6 variants had 7-ethoxycoumarin *O*-deethylase activities. CYP2A6.15 and CYP2A6.18 showed higher activities for safrole 1′-hydroxylation than CYP2A6.1. CYP2A6.25 and CYP2A6.7 had lower safrole hydroxylase activities. CYP2A6.7 had lower flavanone hydroxylase activities, whereas CYP2A6.25 had higher activity toward flavanone. Hydroxyflavanone was metabolized by CYP2A6.25, but was not metabolized by wild-type CYP2A6.1. These results indicate that CYP2A6.25 possessed new substrate specificity toward flavonoids.

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Conflict of Interest

The authors report no conflict of interest.

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