Production of a Human Metabolite of Atorvastatin by Bacterial CYP102A1 Peroxygenase

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Featured Application: The peroxygenase activity of CYP102A1 can be used to prepare atorvastatin drug metabolites and possibly other drugs for biotechnological applications.

Abstract: Atorvastatin is a widely used statin drug that prevents cardiovascular disease and treats hyperlipidemia. The major metabolites in humans are 2-OH and 4-OH atorvastatin, which are active metabolites known to show highly inhibiting effects on 3-hydroxy-3-methylglutaryl-CoA reductase activity. Producing the hydroxylated metabolites by biocatalysts using enzymes and whole-cell biotransformation is more desirable than chemical synthesis. It is more eco-friendly and can increase the yield of desired products. In this study, we have found an enzymatic strategy of P450 enzymes for highly efficient synthesis of the 4-OH atorvastatin, which is an expensive commercial product, by using bacterial CYP102A1 peroxygenase activity with hydrogen peroxide without NADPH. We obtained a set of CYP102A1 mutants with high catalytic activity toward atorvastatin using enzyme library generation, high-throughput screening of highly active mutants, and enzymatic characterization of the mutants. In the hydrogen peroxide supported reactions, a mutant, with nine changed amino acid residues compared to a wild-type among tested mutants, showed the highest catalytic activity of atorvastatin 4-hydroxylation (1.8 min$^{-1}$). This result shows that CYP102A1 can catalyze atorvastatin 4-hydroxylation by peroxide-dependent oxidation with high catalytic activity. The advantages of CYP102A1 peroxygenase activity over NADPH-supported monooxygenase activity are discussed. Taken together, we suggest that the P450 peroxygenase activity can be used to produce drugs’ metabolites for further studies of their efficacy and safety.

Keywords: CYP102A1; atorvastatin; 4-hydroxy atorvastatin; hydrogen peroxide; P450 peroxygenase; NADPH

1. Introduction

Atorvastatin is a cholesterol-lowering drug widely used in treating hypercholesterolemia and preventing cardiovascular disease [1,2]. This statin drug inhibits 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase activity, which catalyzes the rate-limiting step in cholesterol biosynthesis [3]. The atorvastatin can be hydroxylated to 2-OH and 4-OH atorvastatin by cytochrome P450 (CYP or P450) enzyme, CYP3A4, in the human liver [4] (Figure 1, Figure S1). Along with this, atorvastatin’s hydroxylated metabolites have been reported as active metabolites. Among statin drugs, only the atorvastatin has metabolites that can inhibit HMG-CoA reductase equipotent to that of the parent drug [5]. About 70% of the HMG-CoA reductase inhibition achieved with atorvastatin has been observed with its 2-OH and 4-OH atorvastatin [6]. In addition, these two active metabolites have higher efficacy in lowering LDL (low-density lipoprotein) cholesterol when compared with other statins [7]. However, systematic studies have not been performed on the
safety, efficacy, and toxicity of these metabolites, although the metabolites can contribute to atorvastatin’s cholesterol-lowering effect.

![Chemical structures of atorvastatin and its metabolites, 2-OH and 4-OH atorvastatin. The metabolites are made in the human liver by CYP3A4. Bacterial CYP102A1 mutants can make only one metabolite, 4-OH atorvastatin.](image)

Figure 1. Chemical structures of atorvastatin and its metabolites, 2-OH and 4-OH atorvastatin. The metabolites are made in the human liver by CYP3A4. Bacterial CYP102A1 mutants can make only one metabolite, 4-OH atorvastatin.

During the drug development process, which is guided by the U.S. Food and Drug Administration (FDA), the drug metabolites in safety testing (MIST) should be separated to evaluate the safety testing and drug toxicity [8,9]. Although chemical synthesis methods can prepare some drug metabolites of concern, other metabolites such as chiral compounds may not be easily prepared by these methods. Those metabolites can be synthesized by P450 enzymes, including human P450s [10]. Particularly, the CYP102A1 (P450 BM3) from *Bacillus megaterium* demonstrated as a possible biocatalyst for producing human metabolites, which could be useful in fine-chemical biosynthesis and the pharmaceutical fields [11,12]. The CYP102A1 mutants, with new or enhanced catalytic activities toward drugs, could be created by site-directed mutagenesis, random mutagenesis, and rational design [13]. The CYP102A1 was also proposed for industrial application as a potential monooxygenase with high catalytic activities and stabilities [11,14].

The P450s’ typical function is to catalyze the oxidation of a wide range of physiological substrates and foreign chemicals. P450s reductively activate molecular oxygen (O2) by using two electrons transferred to P450s by NAD(P)H via P450 reductase (CPR) [15]. Otherwise, hydrogen peroxide (H2O2) can be used as an oxidant for oxygen activation of P450s via the peroxide shunt pathway [16]. Using peroxides is interesting for monooxygenation reactions catalyzed by P450s as the reaction does not require an electron transfer partner and cofactor NAD(P)H. The low-cost H2O2 can lead to using the P450 peroxygenase in industrial-scale synthesis [17].

Reports found that some of the CYP102A1 mutants obtained via random mutagenesis could catalyze regioselective hydroxylation of atorvastatin to produce 4-OH atorvastatin, which is one of two of the major metabolites in humans, by NADPH-dependent CYP102A1 catalyzed reaction [12] (Figure 1). In this work, we examined the peroxygenase activity of CYP102A1 mutants in having high atorvastatin 4-hydroxylation activity supported by H2O2 and compared this to the mutants’ activities supported by the NADPH regeneration system. We found that the peroxygenase activity of CYP102A1 can be used as a biocatalyst to catalyze the reaction of atorvastatin hydroxylation because CYP102A1 peroxygenase with a low cost has higher activity than that of the NADPH-supported activity.
2. Materials and Methods

2.1. Materials
Atorvastatin, hydrogen peroxide, and glucose oxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-OH atorvastatin and 4-OH atorvastatin were obtained from Toronto Research Chemicals (North York, ON, Canada) Other chemicals and solvents were purchased with the highest grade from commercial suppliers.

2.2. Hydroxylation of Atorvastatin by CYP102A1
The CYP102A1 mutants’ catalytic activity in the hydroxylation of atorvastatin was supported by NADPH regeneration system or hydrogen peroxide. The reaction mixtures include 200 µM of atorvastatin and 0.20 µM CYP102A1 in 100 mM of a potassium phosphate buffer (pH 7.4), and were pre-incubated for 5 min at 37 °C. Hydroxylation of atorvastatin with an NADPH regeneration system (0.5 mM NADP⁺, 10 mM glucose-6-phosphate, and 1.0 IU yeast glucose-6-dehydrogenase/mL) was used to initiate reaction. The reaction mixtures were incubated at 37 °C for 30 min and stopped by 600 µL of ice-cold ethyl acetate.

Quercetin (50 µM) was added as an internal standard to this solution. Then, the mixtures were vortexed for 3 min. After centrifugation (3000 × g, 10 min), aliquots (350 µL) of the ethyl acetate layer were transferred to a clean glass tube and the ethyl acetate was removed under a gentle stream of N₂ gas. Each sample was dissolved in 170 µL of 30% of HPLC mobile phase (see below) and 30 µL was used for the quantitation of the samples.

The atorvastatin’s metabolites were analyzed by HPLC, using a Gemini C18 column (4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA, USA) with the mobile phase A (79.9% water, 20% acetonitrile, and 0.1% formic acid) and the mobile phase B (9.9% water, 90% acetonitrile and 0.1% formic acid). The elution rate was 1.0 mL/min by a gradient pump (LC-20AD; Shimadzu, Kyoto, Japan) with the following gradient: 0–8 min, 30% mobile phase B; 8–13 min, gradually increased to 60% mobile phase B; 13–20 min decreased to 30% mobile phase B; and 20–30 min fixed at 30% mobile phase B. The UV at 260 nm detected the eluent. The CYP102A1 mutants used in this study are shown in Supplementary Table S1.

Calibration standards of atorvastatin and quercetin were constructed from a blank sample (a reaction mixture without substrate and internal standard), nine samples of atorvastatin (2–500 µM), and thirteen samples of quercetin covering 0.2–500 µM (Figure S2). The peak area ratio of atorvastatin to internal standard (quercetin) was linear with respect to the analyte concentration over the range of 0.2–500 µM. When 2.4 volume of ethyl acetate was used to extract atorvastatin and internal standard in buffer solution, the extraction efficiencies of atorvastatin and internal standard were 79% and 74%, respectively, at the concentrations used in the assay. Quantitation of the metabolites was done by comparing the peak areas of each metabolite to mean peak areas of the internal standard (50 µM). The detection limit of 4-OH atorvastatin was 0.20 µM.

2.3. Construction of an Expression Vector for the Heme Domain of CYP102A1 Mutant
The polymerase chain reaction (PCR) was performed using a Thermal Cycler (BioRad, Richmond, CA, USA) and Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). For the expression of the heme domain of M371, the gene-coding heme domain (1–472 residues) was amplified by the PCR with oligonucleotide primers BamHI/XbaI restriction sites: BM3-BamHI forward, 5′-AGCGGATCCATGACAATTAAAGAAATGCC-3′ and BM3-XbaI reverse, 5′-TCTAGACTATTAGCGTACTTTTTAGCAGACTG-3′. The PCR product was resolved on a 1% (v/v) agarose gel, purified, digested with BamHI and XbaI and ligated into a pCWori plasmid [18,19]. The recombinant pCW vector containing M371-heme was expressed in *Escherichia coli* DH5αF’IQ.
2.4. Expression of CYP102A1 Mutants

The plasmids of whole M371 [20,21] and M371-heme were transformed into E. coli DH5α F'-IQ cells and spread on Luria-Bertani (LB) agar plate containing ampicillin (100 µg/mL). The single colony was grown in 5 mL of LB medium containing ampicillin (100 µg/mL) at 37 °C and 170 rpm for 10 h. The cell culture's (1% v/v) aliquots were inoculated in 200 mL of a Terrific Broth (TB) medium with 100 µg/mL ampicillin, 1 mM thiamine, trace elements, 50 µM FeCl₃, 1 mM MgCl₂, and 2.5 mM (NH₄)₂SO₄. The cells were grown at 37 °C and 170 rpm to an OD₆₀₀ of 0.6–0.8. Then, isopropyl-β-D-thiogalactopyranoside (0.50 mM) and δ-aminolevulinic acid (1.0 mM) were added for CYP protein expression. After the cells were grown at 30 °C and 170 rpm for 22 h, the cells were collected by centrifugation (15 min, 5000 × g, 4 °C). The cell pellet was resuspended in 100 mM Tris-HCl (pH 7.6) containing 0.50 mM ethylenediaminetetraacetic acid (EDTA) and 500 mM sucrose, and lysed by sonication (Sonicator, Heat Systems—Ultrasonics, Plainview, NJ, USA). After the lysate was centrifuged at 100,000 × g (90 min, 4 °C), P450 concentrations were examined from the CO-difference spectra using the extinction molecular coefficient: ε = 91 mM⁻¹ · cm⁻¹ [22]. The supernatant of the lysate was used for the catalytic activity assay of the CYP102A1 enzymes.

2.5. LC-MS Analysis

To characterize the major metabolite of atorvastatin produced by the CYP102A1, the reaction mixtures (1 mL) included 0.20 µM whole M371 enzyme and 200 µM atorvastatin in 100 mM potassium phosphate buffer (pH 7.4). The initial reaction was started by the NADPH-generating system at 37 °C for 30 min. The atorvastatin and metabolites were analyzed using Shimadzu LCMS-2010 EV system (Shimadzu, Kyoto, Japan). The metabolites and substrates were separated on a Shim-pack VP-ODS column (2.0 mm i.d. 250 mm; Shimadzu) using a mobile phase with acetonitrile–water–formic acid (70:30:0.1, v/v/v) at a flow rate of 0.16 mL/min. To identify the metabolites, electrospray ionization in positive mode recorded the mass spectra. The interface and detector voltages were 4.4 and 1.5 kV, respectively. The nebulization gas flow was set at 1.5 L/min. The interface, curve desolvation line, and heat block temperatures were 250 °C, 230 °C, and 200 °C, respectively [23].

2.6. Atorvastatin Hydroxylation's Kinetic Parameters and Total Turnover Numbers

For determining the kinetic parameters of CYP102A1 mutants, the reaction mixtures included an atorvastatin substrate at a concentration of 5–200 µM in 100 mM potassium phosphate buffer (pH 7.4) and 0.20 µM CYP102A1. The reaction mixtures were preincubated for 5 min at 37 °C. An aliquot of 10 mM H₂O₂ initiated the reactions, which were performed for 10 min at 37 °C.

To determine total turnover numbers (TTNs) of CYP102A1 mutants, the reaction mixtures included atorvastatin substrate at a concentration of 200 µM in 100 mM potassium phosphate buffer (pH 7.4) and 0.20 µM CYP102A1. The reaction mixtures were preincubated for 5 min at 37 °C. An aliquot of 10 mM H₂O₂ initiated the reactions, which were performed for 30 s, 1, 2, 3, 4, 5, 10, 20, and 30 min at 37 °C.

The kinetic parameters of CYP102A1 catalyzed reactions supported by NADPH were determined by the reaction, including 5–500 µM of atorvastatin in 100 mM potassium phosphate buffer (pH 7.4) and 0.20 µM enzymes. The NADPH-generating systems were added to the initial reaction and the reaction mixtures were incubated at 37 °C for 30 min. The products were extracted with ethyl acetate and analyzed by HPLC as described above. The kinetic parameters were analyzed by GraphPad Prism software (GraphPad Software, San Diego, CA, USA).
2.7. Comparison of Atorvastatin Hydroxylation Activity Supported by External Addition of Hydrogen Peroxide and In Situ Hydrogen Peroxide Generation

For the reactions with externally added hydrogen peroxide, the reaction mixtures included 2 mL total volume including 200 µM atorvastatin in a potassium phosphate buffer (100 mM, pH 7.4) and 0.20 µM CYP102A1. The reaction mixtures were pre-incubated for 5 min at 37 °C. An aliquot of 10 mM H₂O₂ initiated the reactions.

For the reactions with in situ hydrogen peroxide production, the reaction mixtures included 2 mL total volume including 200 µM atorvastatin, 0.20 µM CYP102A1, 4 g/L of glucose, and 10 U/mL of glucose oxidase in a potassium phosphate buffer (100 mM, pH 7.4).

The reactions were performed at 37 °C at 2, 5, 10, 20, 30, 60, and 120 min point time, aliquots of 250 µL reaction mixture were taken and the reactions were stopped by adding 600 µL of ice-cold ethyl acetate for 4-OH atorvastatin measurement.

2.8. Measuring Hydrogen Peroxide Concentration

The hydrogen peroxide concentration was measured by a spectrophotometric assay [24]. The reactions included in situ hydrogen peroxide production, and the reaction mixtures with 2 mL included 200 µM atorvastatin, 0.20 µM CYP102A1, 4 g/L of glucose, and 10 U/mL of glucose oxidase in a potassium phosphate buffer (100 mM, pH 7.4). After the reactions were performed at 37 °C during 2–60 min, aliquots of 100 µL reaction mixture were taken for the hydrogen peroxide measurement. The assay of 1 mL total volume including 100 µL of H₂O₂ production reaction was mixed with 0.28 mM 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 0.5 U/mL of the horseradish peroxidase (HPR) in 100 mM potassium phosphate buffer (pH 7.4). The formation of the green ABTS cation radical was measured spectrophotometrically for 1 min at 420 nm (ε = 36 mM⁻¹ · cm⁻¹) [25].

2.9. The Effect of Cosolvent on Hydroxylation of Atorvastatin Supported by Hydrogen Peroxide

The reaction mixtures included the atorvastatin substrate at a concentration of 200 µM, 0.20 µM CYP102A1, and cosolvent (methanol, ethanol, isopropanol, or acetonitrile glycerol) of 0.5 to 10% (v/v) in a potassium phosphate buffer (100 mM, pH 7.4). The reaction mixtures were pre-incubated for 5 min at 37 °C. An aliquot of 10 mM H₂O₂ initiated the reactions, which were performed for 10 min at 37 °C.

2.10. Spectral Binding Titration

The spectral binding titrations of substrates to the CYP102A1 were determined with a Shimadzu 1601PC Spectrometer at 23 °C, as described previously [26]. The atorvastatin’s binding affinity to four CYP102A1 mutants was determined by titrating 1.0 µM enzyme with the substrate in a total of 1 mL volume in a potassium phosphate buffer (100 mM, pH 7.4). The absorption of the UV-vis spectral difference from 350 nm to 500 nm was recorded after each substrate addition and plotted against the added substrate concentration (0–100 µM). The spectrally determined dissociation constants (Kₐ values) were determined using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

2.11. Statistical Analysis

All experiments were performed three times. The values are presented as means with a standard error of mean (SEM).

3. Results and Discussion

3.1. Hydroxylation of Atorvastatin

At first, we screened the atorvastatin hydroxylation activities of a set of CYP102A1 mutants, which showed high activities toward several drugs supported by NADPH [20,21]. We found some mutants showed higher catalytic activity of atorvastatin 4-hydroxylation when compared to those of our previous work [12]. The mutants M179 (0.89 min⁻¹),
M221 (1.25 min⁻¹), M371 (2.0 min⁻¹), and M387 (0.83 min⁻¹) showed 3.2–7.8-fold higher formation rate of 4-OH atorvastatin than the template M16V2 to make an enzyme library by error-prone PCR (0.26 min⁻¹) (Figure 2). Based on these results, M16V2, M179, M221, M371, and M387 were selected and used for studying atorvastatin hydroxylation activities supported by H₂O₂. Each mutant bore amino acid changes compared to WT CYP102A1, as described in Table S1. Another major metabolite of atorvastatin, 2-OH product, in the human liver, was not found in the CYP102A1-catalyzed reaction.

The mutants M179, M221, M371, and M387 showed 7.5-fold, 7.1-fold, 76.6-fold, and 19.5-fold higher rates of 4-OH atorvastatin formation than M16V2, respectively, when the reaction was performed with the externally added H₂O₂ (10 mM). We found that 10 mM was the most optimal H₂O₂ concentration to support the peroxygenase activity of CYP102A1 (Figure S3). The mutant M371 (1.8 min⁻¹) showed the highest catalytic activity toward atorvastatin among the tested mutants (Figure 2).

M371’s catalytic activities toward atorvastatin did not show large differences between NADPH- and H₂O₂ supported reactions (Figure S4). Based on these results, M371 showed the highest product formation rates in both the NADPH and H₂O₂ dependent reactions. Thus, we selected M371 for constructing the heme domain in an expression vector. The major metabolites and substrates were characterized by the HPLC (Figure 3) and LC-MS (Figure S5) results.

![Figure 2. Activity of atorvastatin 4-hydroxylation catalyzed by CYP102A1 mutants. The reaction mixtures included atorvastatin substrate (200 μM) and 0.20 μM of CYP102A1 in 100 mM of a potassium phosphate buffer (pH 7.4). For NADPH-supported reactions, an NADPH-generating system was used to initiate the reaction, which was incubated for 30 min at 37 °C. For H₂O₂ supported reactions, 10 mM H₂O₂ was used to initiate the reaction, which was incubated for 10 min at 37 °C.](image-url)
Appl. Sci. 2021, 11, x FOR PEER REVIEW 7 of 13

Figure 3. (A) HPLC chromatograms of substrate atorvastatin and its metabolites, 2-OH and 4-OH products. The concentration of each compound was 200 μM. (B) HPLC chromatograms of atorvastatin metabolites produced by whole M371 and M371-heme domain in the presence of H2O2. The hydroxylation of 200 μM atorvastatin by 0.20 μM CYP102A1 (M371 whole enzyme or heme domain) in 100 mM of a potassium phosphate buffer (pH 7.4) was supported by externally added hydrogen peroxide (10 mM) at 37 °C for 10 min. The reaction mixtures’ peaks of HPLC chromatograms were identified by comparing their retention times to those of the following standards: authentic 4-OH atorvastatin (tR = 16.2 min), and atorvastatin (tR = 17.3 min).

3.2. The Kinetic Parameters and TTNs of Atorvastatin Hydroxylation Reactions Supported by Hydrogen Peroxide

The mutant M371, which has both domains of heme and reductase (so-called whole M371), and M371-heme domain were used to determine the kinetic parameters of atorvastatin 4-hydroxylation (Figure 4, Table 1). The kcat value of whole M371 (4.3 min⁻¹) increased 3.3-fold when compared to that of the M371 heme domain (1.3 min⁻¹). The Km values of M371 and M371-heme were 52 μM and 106 μM, respectively. The M371-heme domain showed a 2-fold increased Km value when compared with M371. The catalytic efficiency (kcat/Km) of 4-OH atorvastatin formation by whole M371 and M471-heme domain were 0.083 and 0.012 (min⁻¹·μM⁻¹), respectively. These results showed that whole M371 was more efficient for 4-OH atorvastatin formation than M371-heme (6.9-fold).

Table 1. Kinetic parameters of atorvastatin 4-hydroxylation by whole M371 and M371-heme domain.

| CYP102A1       | kcat (min⁻¹) | Km (μM)  | kcat/Km (min⁻¹·μM⁻¹) |
|----------------|--------------|----------|----------------------|
| whole M371     | 4.3 ± 0.3    | 52 ± 11  | 0.083 ± 0.018        |
| M371-heme domain | 1.3 ± 0.2    | 106 ± 35 | 0.012 ± 0.004        |

Whole M371 and M371-heme were used to determine the TTNs of atorvastatin 4-hydroxylation supported by H2O2 at the reaction times of 30 s, 1, 2, 3, 4, 5, 10, 20, and 30 min (Figure 5). The overall product formation was in the range of 3.2–17.3 TTNs (Figure 5). The whole M371 showed a higher 4-OH atorvastatin formation rate than that of M371-heme during all of the indicated reaction times. In addition, the results showed that 4-OH atorvastatin formation reached a plateau after 10 min of a reaction.
comparing their retention times to those of the following standards: authentic 4-hydrogen peroxide (10 mM) at 37 °C for 10 min. The reaction mixtures' peaks of HPLC chromatograms were identified by whole enzyme or heme domain) in 100 mM of a potassium phosphate buffer (pH 7.4) was supported by

The reaction mixtures included atorvastatin substrate at concentration of 200 µM of whole M371 (#) or heme-domain in 100 mM of a potassium phosphate buffer (pH 7.4) (●). We added 10 mM H₂O₂ to initiate the reaction and the reaction mixtures were incubated for 30 s, 1, 2, 3, 4, 5, 10, 20, and 30 min at 37 °C.

Figure 4. Kinetic parameters of atorvastatin 4-hydroxylation by whole M371 and M371-heme in the presence of H₂O₂. The reactions include 5–200 µM atorvastatin substrate and 0.20 µM of CYP102A1 M371 whole enzyme (#) or heme domain in 100 mM of a potassium phosphate buffer (pH 7.4) (●). We added 10 mM H₂O₂ to initiate the reaction, which was incubated for 5 min at 37 °C.

Figure 5. Total turnover numbers of atorvastatin 4-hydroxylation by CYP102A1 supported by H₂O₂. The reaction mixtures included atorvastatin substrate at concentration of 200 µM and 0.20 µM of whole M371 (#) or heme-domain in 100 mM of a potassium phosphate buffer (pH 7.4) (●). We added 10 mM H₂O₂ to initiate the reaction and the reaction mixtures were incubated for 30 s, 1, 2, 3, 4, 5, 10, 20, and 30 min at 37 °C.

Taken together, the results of kinetic parameters and TTNs with whole enzyme and heme-domain of M371 indicate that the whole protein shows higher catalytic activity than the heme-domain, even if the reductase domain of the whole enzyme does not involve in the peroxygenase activity of the heme-domain. The catalytic activity of M371-heme toward atorvastatin is much lower (~45%) than the whole M371 (Figure 5). This result might be due to a more stable conformation of whole M371 enzyme than that of M371-heme enzyme. This result suggests the reductase domain makes the heme-domain a more functional conformation for its peroxygenase activity.

When we compared the kinetic parameters of whole M371 catalyzed 4-hydroxylation of atorvastatin supported by NADPH and H₂O₂, the kcat value of H₂O₂ supported reaction was 1.4-fold higher than that of the NADPH-supported reaction (Table 1; Table S2). The
K_m value of the H_2O_2-supported reaction was 2.8-fold lower than that of the NADPH-supported reaction. This result means that the H_2O_2-supported reaction showed a higher catalytic efficiency (3.3-fold) than that of the NADPH-supported reaction.

### 3.3. Comparison of Atorvastatin 4-Hydroxylation Activity of CYP102A1 Supported by External Addition and In Situ Generation of Hydrogen Peroxide

A continuous low-level supply or in situ generation of hydrogen peroxide is important for peroxynasenase’s stability [27,28]. The overall productivity can be increased due to enhanced enzyme stability at tailored H_2O_2 generation rates. Here, we compared the peroxynasenase activity of CYP102A1 supported by externally added H_2O_2 (10 mM) or by in situ generation of H_2O_2 via glucose and glucose oxidase.

The catalytic activity of atorvastatin 4-hydroxylation supported by externally adding 10 mM H_2O_2 increased rapidly from a 2–10 min reaction time, after that, 4-OH formation rates reached a plateau (Figure 6). After 30 min, the reaction rate decreased slightly.

![Figure 6](image)

Figure 6. Comparison of external addition of H_2O_2 generation and in situ H_2O_2 generation to support the atorvastatin 4-hydroxylation activity of CYP102A1. The reaction mixtures included atorvastatin substrate at a concentration of 200 μM and 0.20 μM CYP102A1 in 100 mM of a potassium phosphate buffer (pH 7.4). To start the reaction, 10 mM H_2O_2 was externally added to the reaction mixtures (○) or 4 g/L of glucose and 10 U/mL of glucose oxidase were added (●).

When glucose oxidase and glucose were used to produce H_2O_2 continuously, the atorvastatin 4-hydroxylation activity gradually increased from a 2–30 min reaction time. The product formation reached a plateau at 30 min and then increased slightly up to 60 min. The results show that in situ H_2O_2 generation is more suitable for 4-OH atorvastatin formation than that of externally adding H_2O_2 to support the peroxynasenase activity of CYP102A1.

When the concentrations of H_2O_2 with glucose oxidase and glucose were determined under the same experimental conditions with M371, the H_2O_2 concentrations increased to 2.7 mM after 5 min, was constant until 10 min, and then decreased to 1.3 mM after 60 min (Figure S6). These results show the distinctive effects of the two H_2O_2 systems, the external addition and in situ generation, were observed on the atorvastatin 4-hydroxylation activity of CYP102A1.

### 3.4. Effect of Cosolvent on Atorvastatin Hydroxylation Activity Supported by Hydrogen Peroxide

We examined the cosolvent’s effect on the peroxynasenase activity of CYP102A1 toward atorvastatin to find a good solvent system for the atorvastatin hydroxylation activity. Although methanol, ethanol, isopropanol, and acetonitrile showed the 4-OH atorvastatin formation rate generally decreasing (Figure 7A), glycerol gradually increased the 4-OH atorvastatin formation rate when its concentrations increased up to 2.0% (v/v) (Figure 7B).
When the glycerol concentration increased by more than 3%, the activity gradually decreased. The results show the peroxygenase activity of the whole M371 toward atorvastatin reached the highest (5.1 min⁻¹) when 2% glycerol was added in the reaction mixtures.

![Figure 7](image_url)

The cosolvent’s effect on atorvastatin hydroxylation supported by hydrogen peroxide. The reaction mixtures included atorvastatin substrate at 200 μM, 0.20 μM whole M371, and indicated cosolvent in 100 mM of a potassium phosphate buffer (pH 7.4). (A) Cosolvent (methanol, ethanol, isopropanol, and acetonitrile) with 1%, 3%, 5%, and 10% (v/v). (B) Cosolvent: glycerol with 0.5%, 1%, 2%, 3%, 5%, and 10% (v/v).

3.5. Spectral Binding Titration

To examine the differences in the substrate atorvastatin’s binding affinity to mutants M179, M221, M371, and M387, spectral binding titration was performed (Figure 8). Binding of atorvastatin to all of the mutants tested here produced a pronounced Type II spectral shift, with a decrease at 390 nm and an increase at 420 nm, showing an increase in the heme-domain’s low-spin fraction. The mutant’s $K_d$ values ranged between 2.5 and 3.7 μM, although M221 showed a higher $K_d$ value (17 μM) than others. When 4-OH atorvastatin, the product, was added to M371, a modified Type I spectral shift was produced with a decrease at 420 nm and an increase at 390 nm (Figure S7). The $K_d$ value of 4-OH atorvastatin to whole M371 was 6.2 μM.

Although deuterated analogs were not used as internal standards to the analysis of atorvastatin and its metabolite by LC-MS, the kinetic findings in this study should be reliable because commercially obtained 4-OH atorvastatin was also used to confirm the results of subsequent kinetic studies. We found the calibration methods used to quantify the metabolite using quercetin as an internal standard were similar to the direct comparison of external 4-OH atorvastatin standard. The extraction efficiency of 4-OH atorvastatin was 75% under the experimental conditions used in this study.

Drug metabolites in humans produced by P450s are critical for evaluating drug efficacy and safety in the drug discovery and development process. Lower activities of P450 enzymes are suggested for the $H_2O_2$-supported reaction rather than the NADPH-supported reaction, which can be explained by the lack of general acid–base residues in the P450 active sites [17]. This study showed that several CYP102A1 mutants can catalyze human drug metabolites with high peroxygenase activity to produce the human metabolite 4-OH atorvastatin without requiring NADPH, an expensive cofactor [29]. Our results suggest that the catalytic activities of CYP102A1 peroxygenase toward atorvastatin can be improved by enzyme engineering via random mutagenesis and site-directed mutation. The CYP102A1 mutants selected by high-throughput screening [12,20,21] showed high catalytic activity toward atorvastatin. Atorvastatin inhibits HMG-CoA reductase and is a very popular clinical drug used for hypertension and hyperlipidemia. The 4-OH atorvastatin is known as an active metabolite, which is an expensive commercial product. By further improving CYP102A1 peroxygenase activity and stability, the peroxygenase activity can be
used to prepare drug metabolites of atorvastatin and possibly other drugs for industrial purposes.

![Graphs showing dissociation constants](image)

**Figure 8.** Binding titration of atorvastatin to the CYP102A1. The samples for binding titration contained 0–100 μM atorvastatin in 100 mM of a potassium phosphate buffer (pH 7.4) and 1 μM of M179 (A), M221 (B), M371 (C), and M387 (D). Spectrally determined dissociation constants ($K_d$) were also shown.

### 4. Conclusions

In this study, we found a simple enzymatic strategy of CYP102A1 enzymes for a high synthesis efficiency for the 4-OH atorvastatin by using bacterial CYP102A1 peroxygenase activity with hydrogen peroxide. A set of CYP102A1 mutants with high catalytic activity toward atorvastatin were obtained using enzyme library generation, high-throughput screening of highly active mutants, and enzymatic characterization of the mutants. In $H_2O_2$-supported reactions, the mutant M371 among the tested mutants showed the highest catalytic activity of atorvastatin 4-hydroxylation. Improved enzymatic properties of CYP102A1 peroxygenase activity over the NADPH-supported activity were found. This result shows that CYP102A1 peroxygenase activity can catalyze atorvastatin 4-hydroxylation by peroxide-dependent oxidation with high catalytic activity. These results suggest that the peroxygenase activity of CYP102A1 mutants can be developed to produce drugs' metabolites to further study their efficacy and safety.

**Supplementary Materials:** The following are available online at [https://www.mdpi.com/2076-3417/11/2/603/s1](https://www.mdpi.com/2076-3417/11/2/603/s1), Table S1: The amino acid sequences of M16V2 and CYP102A1 mutants, Table S2: Kinetic parameters of atorvastatin 4-hydroxylation by CYP102A1 supported by NADPH and dissociation constants for atorvastatin, Figure S1: Proposed pathway for the atorvastatin metabolism in human, Figure S2: Standard curves of internal standard, quercetin, and atorvastatin, Figure S3: Dependence of atorvastatin 4-hydroxylation by CYP102A1 mutant on the $H_2O_2$ concentration, Figure S4. HPLC chromatograms of atorvastatin and its metabolite produced by CYP102A1 mutant with and without NADPH, Figure S5: LC/MS spectra of atorvastatin and its major metabolite produced by CYP102A1 mutant (M371), Figure S6: Measurement of $H_2O_2$ concentration during atorvastatin 4-hydroxylation by whole M371 via in situ $H_2O_2$ generation, Figure S7: Binding titration of 4-OH atorvastatin to the CYP102A1.
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