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Biosynthesis of Nylon 12 Monomer, ω-Aminododecanoic Acid Using Artificial Self-Sufficient P450, AlkJ and ω-TA

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Received: 25 August 2018; Accepted: 15 September 2018; Published: 18 September 2018

Abstract: ω-Aminododecanoic acid is considered as one of the potential monomers of Nylon 12, a high-performance member of the bioplastic family. The biosynthesis of ω-aminododecanoic acid from renewable sources is an attractive process in the polymer industry. Here, we constructed three artificial self-sufficient P450s (ArtssP450s) using CYP153A13 from *Alcanivorax borkumensis* and cytochrome P450 reductase (CPR) domains of natural self-sufficient P450s (CYP102A1, CYP102A5, and 102D1). Among them, artificial self-sufficient P450 ($CYP_{153A13}BM3_{CPR}$) with CYP102A1 CPR showed the highest catalytically activity for dodecanoic acid (DDA) substrate. This form of ArtssP450 was further co-expressed with ω-TA from *Silicobacter pomeroyi* and AlkJ from *Pseudomonas putida* GPo1. This single-cell system was used for the biotransformation of dodecanoic acid (DDA) to ω-aminododecanoic acid (ω-AmDDA), wherein we could successfully biosynthesize 1.48 mM ω-AmDDA from 10 mM DDA substrate in a one-pot reaction. The productivity achieved in the present study was five times higher than that achieved in our previously reported multistep biosynthesis method (0.3 mM).

Keywords: artificial self-sufficient P450; bioplastics; dodecanoic acid; Nylon 12; ω-aminododecanoic acid

1. Introduction

Production of biochemicals from vegetable oil derivatives (e.g., free fatty acids (FFAs)) have drawn great attention as an alternative means to develop sustainable and green production processes, known as biorefinery [1–4]. Fatty acids and fatty acid derivatives are employed for the production of various polymer intermediates and precursors with broad commercial and pharmaceutical implications, including cosmetics, adhesives, lubricants, surfactants, coatings, biofuels, and anticancer agents [5–7]. In particular, long chain ω-hydroxy fatty acids (ω-OHFAs) contain two functional groups—hydroxy and carboxyl groups—at their ends, and they can therefore be further oxidized to fatty aldehydes, dicarboxylic acids, and oxo-fatty acids. Recently, it was reported that dodecanoic acid (DDA) can be efficiently transformed to ω-amino dodecanoic acid (ω-AmDDA) by utilizing different enzymes like Cytochrome P450 monooxygenases, alcohol dehydrogenases (AlkJ), alkane hydroxylase AlkBGT, Baeyer–Villiger monooxygenases (BVMOs), esterases, and ω-transaminases (ω-TAs) [8–10]. ω-AmDDA is a very important monomer for the synthesis of Nylon 12, along with other aliphatic polyamides. Owing to its extraordinary heat-, abrasion-, chemical-, UV-, and scratch-resistance capabilities, Nylon 12 is frequently used as a coating agent on fuel and braking systems in most passenger cars [9,10]. Usually, Nylon 12 is industrially produced from the monomer ω-laurolactam

through ring opening polymerization, the chemical synthesis of which is initiated by the trimerization of 1,3-butadiene originating from steam cracking (200–300 °C) of crude oil [9,10]. However, this method has raised many environmental concerns. Considering the "green" alternatives, synthesis of ω-amino dodecanoic acid from DDA is a potential approach to address this issue. Moreover, DDA shows relatively high solubility in water compared to other medium- to long-chain fatty acid substrates. Earlier, our group reported that CYP153A13 from *Alcanivorax borkumensis* SK2 efficiently transformed DDA to ω-hydroxy dodecanoic acid (ω-OHDDA) in an in vivo reaction [10–12].

Multistep biocatalytic synthesis often negatively impacts practical industrial applications [13,14]. With increasing number of steps in biocatalytic reactions, the number of generated by-products also increases, many of which may be toxic to cells and make it difficult to separate desirable products. Moreover, the expected expression of multiple recombinant proteins and efficient bioconversion from multistep enzymatic reaction itself is a challenging task [10,15]. Hence, the use of lesser number of recombinant proteins is of immense importance for the biosynthesis of industrially important monomers in a one-pot reaction [16]. Recently, we reported the biosynthesis of ω-AmDDA using the cascade of novel CYP153A, AlkJ, and ω-TA enzymes [10]. In that study, we could achieve more than 87% conversion of ω-OHDDA to ω-AmDDA in a sequential biocatalytic reaction. However, the conversion was negligible in a one-pot reaction (Figure 1a). We therefore envisioned to biosynthesize ω-AmDDA in a single-cell, one-pot cascade reaction. In the present study, we changed the reaction pattern of CYP153A13 from a three-protein system to a single-protein system for ω-hydroxylation of DDA. As CYP153A belongs to the class I CYPs, three component systems are required for the activation of the enzyme (Figure S1). Two additional proteins—putidaredoxin reductase (CamA) and putidaredoxin (CamB)—are related to electron transfer system [10–12]. Self-sufficient P450s, belonging to Class VIII CYPs, are catalytically self-sufficient monooxygenase that contain a heme domain (P450 domain) and a flavin reductase domain (cytochrome P450 reductase (CPR) domain) on a single polypeptide chain ([11,12,17–19]; Figure S1). Recently published findings have shown that the fusion construct of CYP153A with the CPR domain of natural self-sufficient P450s results in the generation of the active form of the enzyme and successfully catalyzes a biocatalytic reaction [11,12,17]. In the present study, we also co-expressed the artificial self-sufficient P450s (ArtssP450s) with an alcohol dehydrogenase (AlkJ) from *Pseudomonas putida* and an omega transaminase (ω-TA) from *Silicibacter pomeroyi* for the synthesis of ω-AmDDA from DDA in a one-pot single-cell reaction (Figure 1b).

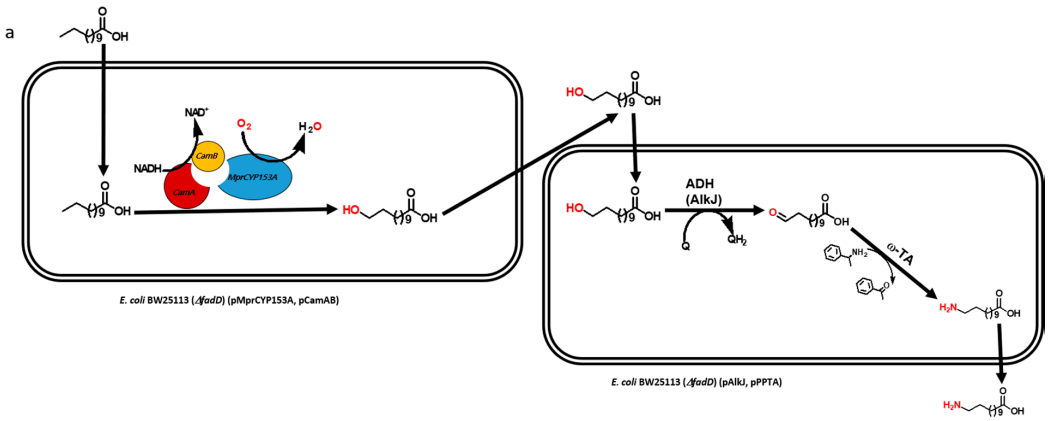

Figure 1. *Cont.*

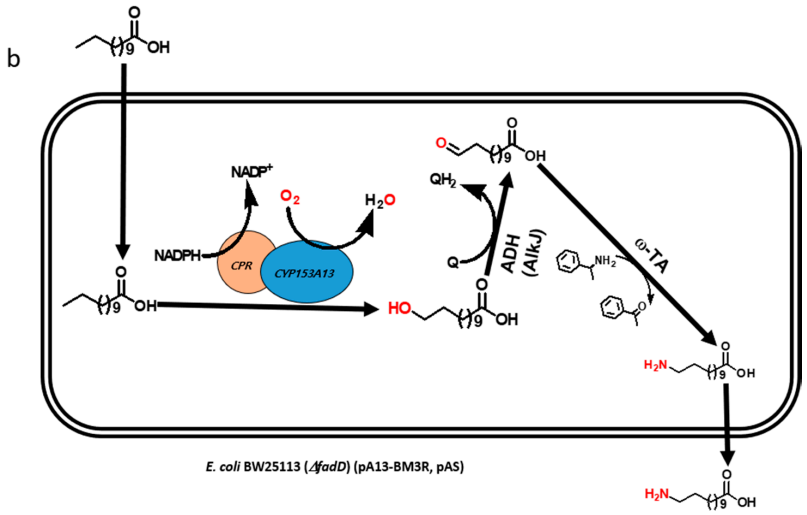

Figure 1. Schematic diagram showing the biosynthesis of ω-amino dodecanoic acid (ω-AmDDA) from dodecanoic acid (DDA) using (**a**) three-protein system (previous study [10]) and (**b**) a single-protein system (this study) for ω-hydroxylation of DDA.

2. Results and Discussion

2.1. Construction and Expression of Artificial Self-Sufficient P450s (ArtssP450s)

CYP153A13 of *Alcanivorax borkumensis* (Abk) was cloned into *Nde*I and *Hind*III restriction sites of pET24ma plasmid vector. The *Hind*III restriction site was chosen to make it a common restriction site to fuse CYP153A13 with CPR domains of CYP102A1, CYP102A5, and CYP102D1 (Figures S2 and S3). After successful cloning of CYP153A13, CYP153A13-containing plasmid was used again to make artificial self-sufficient fusion construct of CYP153A13 and CPRs in the same vector. All three CPR domains were amplified; RE digested and ligated into *Hind*III and *Not*I sites for CYP102A5 and CYP102D1 and into *Hind*III and *Xho*I sites for CYP102A1. To remove *Hind*III sites within the CPR domain of CYP102A1, the 243th alanine residue DNA codon was changed from GCT to GCA by utilizing overlap extension PCR method. Next, all the ArtssP450s were confirmed by sequencing and nomenclatured newly as $Abk_{CYP153A13}BM3_{CPR}$ (for CYP102A1 CPR), $Abk_{CYP153A13}Bc21_{CPR}$ (for CYP102A5 CPR), and $Abk_{CYP153A13}Sav_{CPR}$ (for CYP102D1 CPR) on the basis of their gene source (See Materials and Method). All three different CPRs were chosen from three different natural self-sufficient P450s based on previous reports. Among them, BM3 is a well reported self-sufficient P450, and its CPR domain is frequently used for making artificial constructs [11,12,17]. The self-sufficient P450 of Bc21 (CYP102A5) has been reported to possess the highest turnover rates among P450s [19]. In addition, a codon-optimized Sav (CYP102D1) has been reported to show similar catalytic activity for saturated and unsaturated fatty acids as that of other members of the CYP102A family [20].

After successful construction of ArtssP450s, the expression of all recombinant proteins was tested at three different temperature (20, 25, and 30 °C) and three different isopropyl-thio-β-D-galactopyranoside (IPTG) concentrations—0.01, 0.1, and 0.5 mM—in terrific broth (TB) bacterial expression media. All three proteins showed better expression at 20 °C with 0.1 mM IPTG concentration. The protein expression was confirmed through SDS-PAGE gel analysis (Figure S4). SDS-PAGE analysis demonstrated clear protein expression band at expected size (~120 kDa). Additionally, the conventional carbon monoxide (CO)-differential spectral assay with sodium dithionite was used in cell-free extracts as a standard method to determine the concentration of active P450. The characteristic absorbance at 450 nm for CO complexed to the reduced ferrous state of P450 was observed (Figure 2), corroborating their active expression. The amount of active P450 was 1.51, 2.25, and 0.75 nmol mL^{-1} for $Abk_{CYP153A13}BM3_{CPR}$, $Abk_{CYP153A13}Bc21_{CPR}$, and $Abk_{CYP153A13}Sav_{CPR}$, respectively. These results were well correlated with the reported titer range of the active P450 expression (0.6–2.33 nmol mL^{-1}) in *E. coli* system [18–21].

It has also been reported that, in many cases, the co-expression of CPRs lead to formation of high titers of active P450 [21]. In the CO-binding analysis, $Abk_{CYP153A13}Bc21_{CPR}$ construct seemed to be the most active, being nearly three times higher than $Abk_{CYP153A13}Sav_{CPR}$ and two times higher than $Abk_{CYP153A13}BM3_{CPR}$.

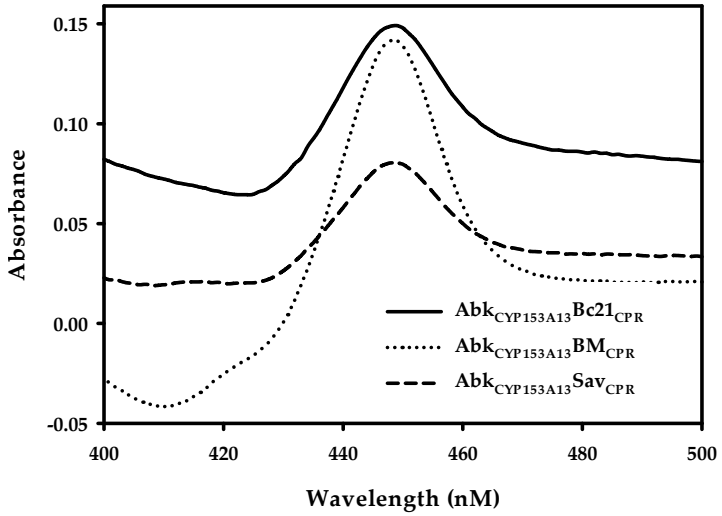

Figure 2. Carbon monoxide (CO)-binding analysis of ArtssP450s. $Abk_{CYP153A13}$, *Alcanivorax borkumensis* CYP153A13; $Abk_{CYP153A13}BM3_{CPR}$, $Abk_{CYP153A13}$ fused with BM3 (CYP102A1) cytochrome P450 reductase (CPR); $Abk_{CYP153A13}Bc21_{CPR}$, $Abk_{CYP153A13}$ fused with *Bacillus cereus* (CYP102A5) CPR; and $Abk_{CYP153A13}Sav_{CPR}$, $Abk_{CYP153A13}$ fused with *Streptomyces avermitilis* (CYP102D1) CPR.

2.2. Production of ω-OHDDA Using ArtssP450s

It has been reported that $Abk_{CYP153A13}$ exhibits better ω-OHDDA productivity among CYP153A13, CYP153A33, and CYP153A35 in an in vivo biocatalytic reaction employing three protein system [11]. In order to know the ω-OHDDA productivity of newly constructed ArtssP450s in a single protein system, all three enzymes were tested with 10 mM DDA substrate in a whole-cell reaction. Additionally, a control whole-cell reaction was performed using $Abk_{CYP153A13}$ in CamAB system. The initial specific rates of ω-hydroxylation were 0.10, 0.09, and 0.037 mmol/g_{CDW}/h for $Abk_{CYP153A13}BM3_{CPR}$, $Abk_{CYP153A13}Bc21_{CPR}$, and $Abk_{CYP153A13}Sav_{CPR}$, respectively. Accordingly, 24-h reactions resulted in 3.95, 2.64, and 1.40 mM ω-OHDDA, respectively (Figure 3). Although the initial specific rates of ω-hydroxylation and production of ω-OHDDA after 24 h were better for $Abk_{CYP153A13}$ in CamAB system (0.16 mmol/g_{CDW}/h and 6.21 mM, respectively) (Data not shown), we used single-protein system due to its obvious advantages. Among ArtssP450s, the initial specific rate of $Abk_{CYP153A13}BM3_{CPR}$ and $Abk_{CYP153A13}Bc21_{CPR}$ were very close. Unexpectedly, the highest productivity (3.95 mM) was observed in $Abk_{CYP153A13}BM3_{CPR}$. As $Abk_{CYP153A13}Bc21_{CPR}$ showed the highest amount of active P450 in CO-binding spectrum analysis, it was estimated that the productivity would also be the highest for $Abk_{CYP153A13}Bc21_{CPR}$ (Figure 2). However, the published findings have demonstrated that BM3 natural self-sufficient P450 (CYP102A1) shows the highest catalytic activity among all P450s [20]. Moreover, CamAB system showed better ω-OHDDA productivity than ArtssP450 systems developed in the present study. Despite these findings, the use of ArtssP450s for ω-OHDDA biosynthesis was of utmost importance in consideration of the one-pot biocatalytic cascade reaction in order to get the final product of this study, i.e., ω-AmDDA (Figure 1b). As $Abk_{CYP153A13}BM3_{CPR}$ system showed the highest ω-OHDDA productivity among all the ArtssP450 constructs, it was further used to produce ω-AmDDA from DDA (Figure 1).

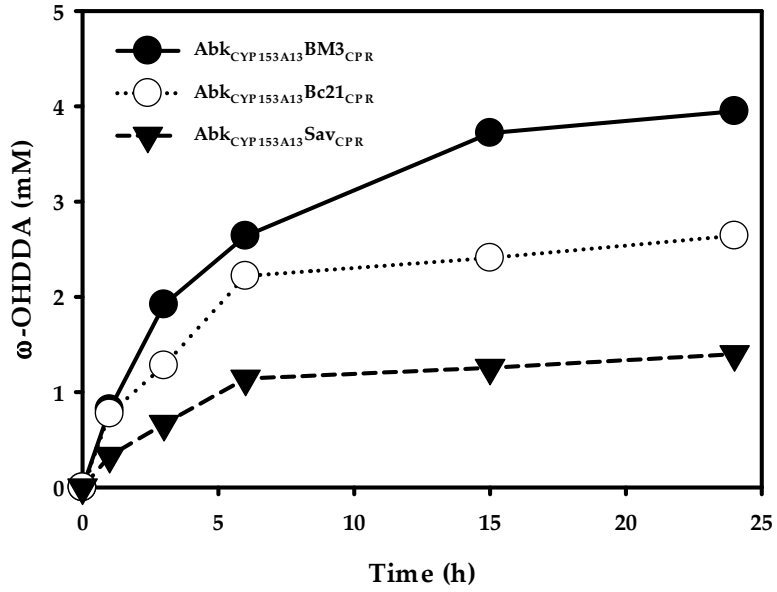

Figure 3. ω-hydroxy dodecanoic acid (ω-OHDDA) production by ArtssP450s. Reaction conditions: substrate concentration, 10 mM; volume, 10 mL in 100 mL flask; temperature, 30 °C; cell type, BW25113 (ΔfadD, DE3) containing pA13-BM3R, pA13-Bc21R, pA13-SavR; cell OD_{600}: 30 and buffer, 100 mM potassium phosphate buffer (pH 7.5) with 1% (w/v) glucose.

2.3. Biosysnthesis of ω-AmDDA in One-Pot Reaction

2.3.1. Co-expression of Abk$_{CYP153A13}$BM3$_{CPR}$ with AlkJ and Sp ω-TA

The production of ω-AmDDA from DDA in a one-pot reaction requires co-expression of Abk$_{CYP153A13}$BM3$_{CPR}$ with AlkJ and ω-TA. In this reaction, the DDA substrate is initially converted to ω-OHDDA by Abk$_{CYP153A13}$BM3$_{CPR}$. AlkJ then catalyzes the transformation of ω-OHDDA to ω-OxoDDA, which is further transaminated by ω-TA to yield ω-AmDDA. Therefore, Abk$_{CYP153A13}$BM3$_{CPR}$ was co-expressed with AlkJ and ω-TA at 20 °C using 0.1 mM IPTG induction in TB media. The expression of all three proteins in the soluble form was confirmed by SDS-PAGE gel analysis (Figure 4). Next, the co-expressed cells were used in a whole-cell reaction to produce ω-AmDDA from DDA in a one-pot reaction. The reaction was initiated by adding 5 mM DDA (stock in dimethyl sulfoxide (DMSO), 5% (v/v) final concentration), 0.1 mM pyridoxal-5′-phosphate (PLP), and 40 mM benzylamine using co-expressed cells (0.3 g_{DCW}/mL) in 100 mM Tris-HCl buffer. The reaction mixture was incubated at 35 °C and 200 rpm. After 6 h, 0.8 and 0.3 mM ω-OHDDA and ω-AmDDA, respectively, were detected (data not shown). This can be explained by the fact that Abk$_{CYP153A13}$BM3$_{CPR}$ transformed DDA to ω-OHDDA. AlkJ converted this ω-OHDDA to ω-OxoDDA, which was utilized by Sp ω-TA to form ω-AmDDA. Albeit with low conversion percentage, the successful biotransformation of DDA to ω-AmDDA encouraged us to further improve the productivity by optimizing different parameters.

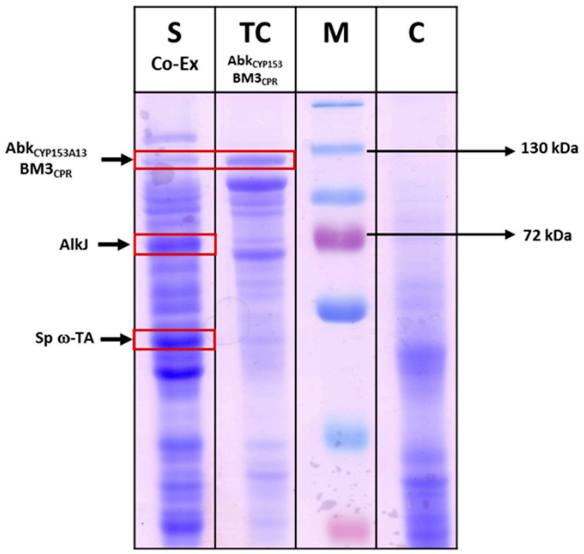

Figure 4. SDS-PAGE analysis of recombinant *E. coli* expressing Abk$_{CYP153A13}$BM3$_{CPR}$ (~120 kDa), AlkJ (~61.2 kDa), and Sp ω-TA (~52 kDa) co-expression. C: control (*E. coli* BW25113(DE3) ΔfadD cells without any plasmid were used as control); M: Marker; TC: total cells; SP: soluble proteins. Protein expression was carried out using 0.1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) at 20 °C and 200 rpm for 16 h.

2.3.2. Effect of Benzylamine (BA) on ω-OHDDA Productivity

The selection of a suitable amino donor is of immense importance to enhance the yield of ω-TA reaction. Organic syntheses by ω-TAs may suffer from unfavorable reaction equilibrium due to insufficient amount of amino donor [16]. To maximize the productivity of ω-TAs, shifting the equilibrium to the product using higher concentration of amino donor is extremely important [22]. Previously, while synthesizing ω-AmDDA from ω-OHDDA using AlkJ and Sp ω-TA, we found benzylamine (BA) as the best amino donor [10]. It has been reported that the performance of P450 employed in the amination cascade is influenced by the amino donor used [23]. Therefore, it was presumed that the productivity of ω-OHDDA might be hindered due to excess amount of BA as amino donor for ω-TA in the one-pot reaction demonstrated herein. Before optimizing ω-AmDDA productivity, the biosysnthesis of ω-OHDDA by Abk$_{CYP153A13}$BM3$_{CPR}$ was tested in 100 mM (pH 7.5) potassium phosphate buffer and Tris-HCl buffer using five different concentrations of BA. It was demonstrated that 0–5 mM BA had negligible effect on the production of ω-OHDDA in both types of buffers, but the productivity was better in the phosphate buffer. Moreover, 50 mM BA had remarkable effect on ω-OHDDA in both the cases (Figure 5). This could be explained by the interception of the hydrogen-borrowing step ($NADP^+$/NADPH) of the cofactors in endogenous *E. coli* enzyme systems, which may lead to imbalanced regeneration of the cofactors [23].

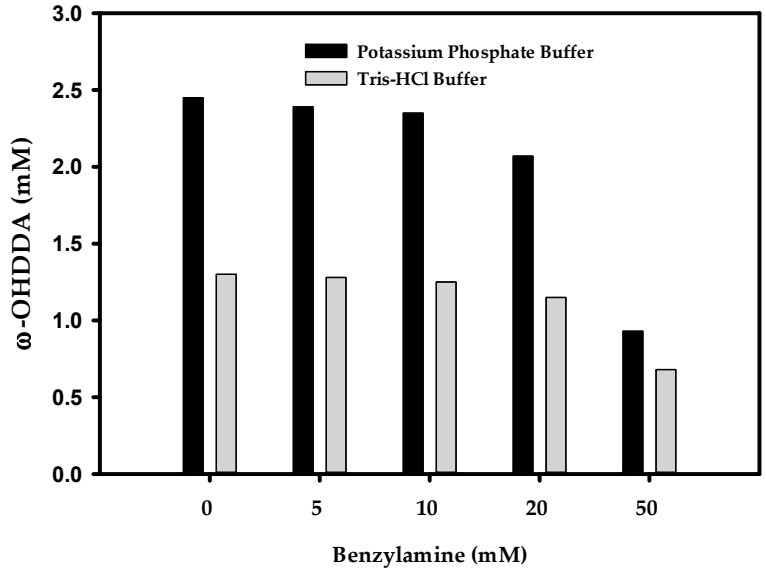

Figure 5. Effect of BA on ω-OHDDA productivity. Reaction conditions: substrate concentration: 10 mM; volume: 10 mL in 100 mL flask; temperature: 35 °C; cell type: BW25113 (ΔfadD, DE3) containing pA13-BM3R; cell OD_{600}: 30 and buffer, 100 mM (pH 7.5) with 1% (w/v) glucose; reaction time: 5 h.

We have previously reported that ω-TA enzyme is more active in Tris-HCl buffer with alkaline pH [10]. In the present study, it was revealed that ArtssP450s did not show any activity for ω-hydroxylation of DDA in an alkali pH (>8) (data not shown). Therefore, further optimization of ω-AmDDA production in a one-pot reaction was continued in 100 mM Tris-HCl buffer (pH 7.5), which seemed to be a convenient reaction condition for all the enzymes.

2.3.3. Optimization of ω-AmDDA Biosynthesis

Finally, in order to check the optimum productivity of ω-AmDDA in a one-pot reaction, cells (0.3 g_{CDW}/mL) co-expressing $Abk_{CYP153A13}BM3_{CPR}$, AlkJ, and Sp ω-TA were used in various concentration of BA (0–50 mM) in a whole-cell reaction. The reaction was initiated by adding 10 mM DDA (stock in DMSO, 5% (v/v) final concentration), 0.1 mM PLP, and 20 mM benzylamine. The reactants were incubated at 35 °C and 200 rpm. After 3 h of whole-cell reaction, the amount ω-AmDDA was 0.03, 0.07, 0.15, and 0.55 mM when the BA was used in the reaction was 0, 5, 10, and 20 mM, respectively. In particular, it was observed that the amount of ω-OHDDA after 3 h was 1.21, 1.10, 0.95, and 0.82 mM, respectively, with the subsequent concentration of used BA. The production of both ω-AmDDA and ω-OHDDA increased up to 5 h, and the amount of ω-AmDDA was 0.10, 0.20, 0.36, and 0.84 mM, respectively, whereas the amount of ω-OHDDA was 2.35, 2.22, 1.98, and 1.64 mM, respectively, for the corresponding amount of BA used. Interestingly, the biosynthesis of ω-AmDDA was increasing over the 15 h reaction period (Figure 6), while the production of ω-OHDDA dropped after 5 h. The whole-cell reaction yielded 0.17, 0.33, 1.03, and 1.17 mM of ω-AmDDA and 1.87, 1.61, 1.12, and 0.56 mM of ω-OHDDA after 15 h when 0, 5, 10, and 20 mM BA was used, respectively (Figure 6). The present study demonstrated that the use of 20 mM BA as an amino donor in a one-pot whole-cell reaction gave the best productivity for the synthesis of ω-AmDDA from DDA substrate. Moreover, the best whole-cell reaction condition was continued up to 24 h. It was found that 1.48 mM ω-AmDDA was biosynthesized from 10 mM DDA, which is five times more than our last report [10]. Whole-cell reaction of *E. coli* cells expressing MprCYP153A/CamAB for ω-hydroxylation of DDA and *E. coli* cells expressing AlkJ/mll1207 ω-TA resulted in the generation of ~0.3 mM ω-AmDDA from DDA in a 24 h reaction [10].

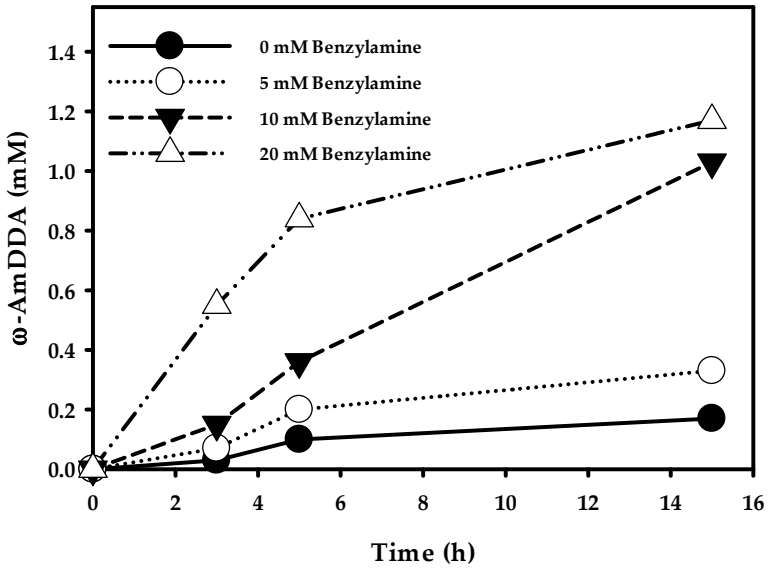

Figure 6. Biosysnthesis of ω-AmDDA from DDA using co-expressed $Abk_{CYP153A13}BM3_{CPR}$, AlkJ, and Sp ω-TA enzymes. Reaction conditions: substrate concentration: 10 mM; volume: 10 mL in 100 mL flask; temperature: 35 °C; cell type, BW25113 (ΔfadD, DE3) containing pA13-BM3R and pAS; cell OD_{600}: 30; cofactor: 0.1 mM pyridoxal-5′-phosphate (PLP); amino donor: 20 mM benzylamine and buffer, 100 mM Tris-HCl buffer (pH 7.5) with 1% (w/v) glucose.

It has been reported that AlkJ from *Pseudomonas putida* GPo1 shows thermal denaturation at moderate temperatures, and T_{stab}—the temperature where the enzyme shows 50% residual activity—was found to be 34 °C [24]. Thus, biotransformations carried out at lower temperatures could have resulted in improved overall yield in the present study. However, the major challenge in one-pot biotransformations employing more than one protein—as demonstrated in the present study—is the satisfactory performance of all proteins involved. In these multiprotein biotransformations, not every protein can be optimally functional in the given set of reaction parameters. Hence, there is always a compromise involved in yield of final product and the optimal performance of the proteins employed in the multiprotein biotransformations. In the addition, the final outcome of multiprotein biotransformations is severely compromised when proteins exhibit differential performance at specific reaction conditions, such as temperature and/or reaction pH. It should also be noted that whole-cell biocatalysts are more stable than their purified counterparts. Nevertheless, in our previously reported study [10], it was observed that all of the initially added ω-OHDDA (5 mM) was consumed within 1 h when whole-cell reaction was performed at 35 °C, implying that AlkJ activity was enough to carry out transformation of ω-OHDDA.

It has been well reported that the *fadL* is required for the transport of long chain fatty acids in *E. coli* [25,26]. Although overexpression of *fadL* was one of the possible approaches to improve the mass transfer of DDA substrate across cell membrane in the present study, it could have adversely affected the expression of other proteins. As expression of a recombinant protein may impart a metabolic burden on the host microorganism [27], the primary object of multiprotein, one-pot biotransformations should be the use of lesser number of recombinant proteins to achieve the synthesis of a desired product. Recently, Janßen et al. [28] reported the improved transfer of long-chain fatty acids in *fadL*-overexpressed *E. coli* cells. However, a recently reported study by our group [10] demonstrated that *E. coli* cells can satisfactorily uptake DDA without overexpression of *fadL*.

3. Materials and Methods

3.1. Chemicals and Media

All chemicals such as DDA, ω-OHDDA, ω-AmDDA, dimethyl sulfoxide (DMSO), IPTG, 5-aminolevulinic acid (5-ALA), *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), *N*-Methyl-*N*-(trimethylsily) trifluoroacetamide (MSTFA), PLP, benzylamine, and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform was obtained from Junsei (Tokyo, Japan). Bacteriological agar, Luria-Bertani (LB) broth, and terrific broth (TB) media were bought from BD Difco (Franklin Lakes, NJ, USA). All chemicals used in this study were of analytical grade.

3.2. Artificial Self-Sufficient P450 Construction and Gene Manipulation

All the bacterial strains and plasmid vectors used in this study are listed in Table 1. *Alcanivorax borkumensis* SK2 (KACC no. 12864) was procured from the Korean Agricultural Culture Collection (KACC, Jeonju, Korea). Genomic DNA was extracted from lyophilized commercial cell stock using a kit (G-Spin™ Genomic DNA Extraction Kit for bacteria (iNtRON Biotechnology, Suwon, Korea)). The gene encoding CYP153A13 (GI: CAL15649.1) from *A. borkumensis* SK2 was amplified by PCR with oligonucleotides (Table S1). After restriction digestion and ligation with T4 DNA ligase, the plasmid was utilized to transform competent *E. coli* DH5α cells. Successful cloning was confirmed by DNA sequencing. Construction of artificial self-sufficient fusion proteins (pA13-BM3R, pA13-Bc21R, and pA13-SavR) with CYP153A13 and the reductase domains (CPRs) of self-sufficient P450s (CYP102A1 from *Bacillus megaterium*, CYP102A5 from *Bacillus cereus*, and CYP102D1 from *Streptomyces avermitilis*) were performed following previously described procedures [18]. CPR domains of self-sufficient P450s were analyzed using online bioinformatics tools Pfam 31.0 site (http://pfam.xfam.org/). Gene synthesis and codon optimization for *E. coli* codon preferences was performed by Cosmo Genetech (Cosmo Genetech, Seoul, Korea).

Table 1. Plasmids and strains used in this study.

Plasmids/Strains	Description	Reference
Plasmids		
pET24ma	P15A ori lacI T7 promoter, KmR	[10]
pETDuet-1	pBR322 ori lacI T7 promoter, AmpR	Novagen
pA13	pET24ma encoding CYP153A13 ($Abk_{CYP153A13}$)	[11]
pA13-BM3R	pET24ma encoding $Abk_{CYP153A13}BM3_{CPR}$	This study
pA13-Bc21R	pET24ma encoding $Abk_{CYP153A13}Bc21_{CPR}$	This study
pA13-SavR	pET24ma encoding $Abk_{CYP153A13}Sav_{CPR}$	This study
pAlkJ	pET24ma encoding AlkJ	[10]
pPPTA	pETDuet-1 encoding Sp ω-TA	[10]
pAS	pETDuet-1 encoding Sp ω-TA and AlkJ	This study
***E. coli* strains**		
DH5α	F^-, *endA1*, *gln*V44, *thi*-1, *rec*A1, *rel*A1, *gyrA*96 *deo*R, *supE*44, Φ80d*lacZ*ΔM15 Δ(*lacZ*YA$^-$-*arg*F)-U169, *hsd*R17(r_K^- m_K^+), λ^-	[29]
BW25113(DE3)	rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 λ(DE3)	[11]
DL	BW25113(DE3) ΔfadD	[10]
A13	DL carrying pA13	This study
A13-BM3R	DL carrying pA13-BM3R	This study
A13-Bc21R	DL carrying pA13-Bc21R	This study
A13-BSavR	DL carrying pA13-BSavR	This study
AlkJ-PPTA	DL carrying pAlkJ and pPPTA	This study
BM-AS	DL carrying pA13-BM3R and pAS	This study

3.3. Expression of Enzymes

E. coli BW25113 (DE3) ΔfadD strain [10] was utilized for the biotransformation studies, wherein fatty acid degrading β-oxidation pathway was blocked. Plasmid DNA were transformed into host strains using standard heat shock method. Transformants were selected based on their antibiotic resistance [30]. Transformants were grown overnight at 37 °C in 10 mL LB medium containing 50 μg/mL of kanamycin (for pA13-BM3R, for pA13-Bc21R, and for pA13-SavR) and/or 100 μg/mL ampicillin (for pAS, pAlkJ, and pPPTA). The seed cultures were added to expression flask into 1/3 ratio. All artificial self-sufficient P450 protein expression were carried out in 200 mL of Terrific Broth in a 1 L flask. They were cultured at 37 °C until cell concentration reached an OD_{600} of 0.6–0.8. The induction was performed by adding 0.1 mM IPTG, 0.5 mM 5-ALA as heme precursor, and 0.1 mM $FeSO_4$, and cells were grown at 20 °C for 16 h. The expression of Sp ω-TA/AlkJ with or without artificial self-sufficient P450 was carried out in 200 mL of LB medium in 1 L flasks. Induction was performed by adding 0.1 mM IPTG, and cells were allowed to grow for 16 h at 20 °C.

After 16 h, cells were harvested by centrifugation (4000 rpm, 20 min, 4 °C), washed with phosphate-buffered saline (PBS), and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 1% (w/v) glucose.

3.4. CO-Binding Assay and Gel Electrophoresis

The expressed proteins were subjected to SDS-PAGE and spectrophotometric analysis in order to measure the CO-binding activity. UV absorption spectra of CO-bound artificial self-sufficient P450 proteins after sodium dithionite reduction were measured by Cary 100 UV-Vis spectrometry (Agilent Technologies, CA, USA) by wavelength scan from 400 to 500 nm. The concentration of P450 was measured by CO-binding affinity using an extinction coefficient of 91.9 mM^{-1} cm^{-1} at 450 nm. SDS-PAGE (Bio-Rad Laboratories, Inc, Hercules, CA, USA.) was carried out with 12% polyacrylamide gel as described elsewhere [31]. Proteins were visualized by Coomassie® brilliant blue R-250 staining.

3.5. Biotransformation

Biotransformation of FFAs to ω-OHFAs was performed according to the previously reported method [10]. For the biotransformation of ω-OHDDA acid to ω-AmDDA, *E. coli* BM-AS (Table 1) cotransformed strain was grown and harvested as described above. The cell pellets were resuspended in 100 mM Tris-HCl buffer (pH 7.5 and 8.0), and biotransformation (300 mg_{CDW}/mL) was performed at 35 °C at 150 rpm in a shaking incubator following our previous published protocols [10]. The whole-cell reaction was started by the addition of ω-OHDDA (stock in DMSO, 5% (v/v) final concentration).

To assess the biotransformation of DDA to ω-OHDDA by ArtssP450s, whole cell reaction was performed according to the previously reported method [10]. Briefly, induced cells expressing ArtssP450s in a single protein system were grown for 16 h at 20 °C. Cells were harvested by centrifugation (4000 rpm, 20 min, 4 °C) and washed with PBS. To initiate the biotransformation, resting cells (300 mg_{CDW}/mL), DDA (10 mM; stock in DMSO, 5% (v/v) final concentration) and buffer (Potassium phosphate; 100 mM, pH 7.5) with 1% (w/v) glucose were added to a 100 mL flask to a final volume of 10 mL. This reaction mixture was incubated at 30 °C and 200 rpm for 24 h. For one-pot biotransformation of DDA to ω-AmDDA, *E. coli* BM-AS (Table 1) cotransformed strain was grown and harvested as described above. Biotransformation was initiated by adding resting cells (300 mg_{CDW}/mL), DDA (10 mM; stock in DMSO, 5% (v/v) final concentration) and buffer (Tris-HCl; 100 mM, pH 7.5 and 8.0) to a 100 mL flask to a final volume of 10 mL. This reaction mixture was incubated at 35 °C and 150 rpm for 24 h.

3.6. Product Identification and Quantification

Whole-cell reactions of both the biotransformations, i.e., DDA to ω-OHDDA and DDA to ω-AmDDA, were stopped and acidified with 6 M HCl to pH 2.0. The substrates and products

of DDA biotransformation were extracted with an equal volume of chloroform (200 µL) after vigorous vortexing for 1 min (Table S2). After centrifugation, the extracted sample in chloroform (bottom layer) was transferred to a new microcentrifuge tube for derivatization. These samples were transformed to their trimethylsilyl (TMS) derivatives by incubation with an excess of BSTFA at 50 °C for 20 min. In the case of biotransformation generating ω-AmDDA, the acidified reaction samples (with 6 M HCl) were centrifuged, and the supernatant was dried in a vacuum concentrator. After complete drying, the supernatant was dissolved again to the original volume with pyridine. The sample was then mixed with an equal volume of MSTFA by vigorous vortexing for 1 min and converted to the TMS derivatives by incubation at 50 °C for 20 min.

Analytical conditions for free fatty acids and their derivatives using gas chromatography are well established and reported in our previously published reports [4,7,10–12,20]. Quantitative analysis was performed using a gas chromatography instrument with a flame ionization detector (GC/FID) fitted with an AOC-20i series auto sampler injector (GC 2010 plus Series, Shimadzu Scientific Instruments, Kyoto 604-8511, Japan). Two-microliter samples were inserted by split mode (split ratio 20:1) and examined by means of a nonpolar capillary column (5% phenyl methyl siloxane capillary 30 m × 320 µm i.d., 0.25-µm film thickness, HP-5). The oven temperature program for fatty acid analysis was 50 °C for 1 min, an increase by 10 °C/min to 250 °C, and hold for 10 min. The inlet temperature was 250 °C, and the detector temperature was 280 °C. The flow rate of the carrier gas (He) was 1 mL/min, and the flow rates of H_2, air, and He in FID were 45 mL/min, 400 mL/min, and 20 mL/min, respectively. For the analysis of DDA and ω-OHDDA, commercial decanoic acid was used as internal standard. Internal standard was added after stopping the reaction and before the centrifugation step of chloroform extraction. For ω-AmDDA analysis, the oven temperature program was modified. The initial oven temperature was 90 °C, which was then increased by 15 °C/min to 250 °C, holding at this temperature for 5 min. Internal standard was not used. As chloroform extraction was not carried out in this analysis, there was presumably no loss of product and substrate as in the chloroform extraction. Pyridine was used for dilution of the product after vacuum evaporation of buffer–water, and dilution factor was considered while quantifying the product yield. Products of the biotransformations were confirmed by comparing the GC chromatograms with authentic references (Figures S5–S8).

4. Conclusions

In summary, we constructed artificial self-sufficient P40s by fusing CYP153A13 with various CPR domains—CYP102A1, CYP102A5, and CYP102D1—and tested them for the hydroxylation of DDA to ω-OHDDA. Furthermore, a single-cell system was generated for the biosynthesis of Nylon 12 monomer ω-AmDDA in a one-pot reaction by co-expressing the best artificial self-sufficient P40 generated (i.e., $Abk_{CYP153A13}BM3_{CPR}$), AlkJ, and Sp ω-TA enzymes. This highly efficient biocatalytic cascade produced ω-AmDDA in an easy and cost-effective way. It is worth emphasizing that the productivity of the biocatalytic cascade reported herein was five times higher than that of our previously reported sequential cascade method [10].

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/9/400/s1, Figure S1: Electron transportation system bacterial CYP. A. class I, and B. class VIII; Figure S2: Co-expression plasmid diagram for the synthesis of ω-AmDDA; Figure S3: Schematic diagram of ArtssP450s construction strategy using restriction sites into pET24ma vector; Figure S4: SDS-PAGE analysis of recombinant *E. coli* expressing ArtssP450s (~120 kDa). C: control; M: marker; TC: total cells; SP: soluble proteins. Protein expression was carried out using 0.1 mM IPTG, 0.1 mM $FeSO_4$, and 0.5 mM 5-ALA at 20 °C and 170 rpm; Figure S5: GC chromatogram of the chemical standard of DDA. The peak at 13.5 min is the internal standard decanoic acid; Figure S6: GC chromatogram of the chemical standard of ω-AmDDA. The peak at 13.5 min is the internal standard decanoic acid; Figure S7: GC chromatogram of authentic ω-AmDDA and reaction mixture of DDA to ω-AmDDA; Figure S8: GC/MS analysis of authentic ω-AmDDA and reaction mixture of DDA to ω-AmDDA; Table S1: Primers used for the construction of ArtssP450s; Table S2: Retention time of the substrates and products by gas chromatography.

Author Contributions: H.Y., and T.C. designed the experiments of the project. M.M.A. carried out the research works as part of his PhD project. H.Y. supervised the whole studies reported in the manuscript. M.M.A. wrote the manuscript. M.D.P. revised this manuscript. H.J., and S.S. assisted in experimental tools.

Funding: This research was funded by the Ministry of Trade, Industry and Energy of South Korea (MOTIE, Korea) under the Industrial Technology Innovation Program, Grant Nos. 10062550 and 10076343.

Conflicts of Interest: The authors declare no financial or commercial conflict of interest.

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