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Protein engineering of toluene monooxygenases for synthesis of hydroxytyrosol

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ABSTRACT

Hyroxytyrosol (HTyr), an important phenol present in olives, stands out as a compound of high added value due to its exceptional antioxidant, antimicrobial and anticarcinogenic activities. This work describes the synthesis of HTyr via double hydroxylation of 2-phenylethanol (PEA) employing toluene monooxygenases (TMOs) as biocatalysts. Wild-type TMOs were initially evaluated for their ability to oxidise PEA and structurally-related substrates, providing better understanding of the factors responsible for controlling the regiospecificity. Both the length of the alkyl side chain and the presence of the hydroxyl group were found to influence the activity, possibly by interfering with the substrates' entrance into the active site. Directed evolution of toluene-4-monooxygenase of *Pseudomonas mendocina* KR1 led to the discovery of variant TmoA S395C with a 15-fold increase in PEA hydroxylation rate. Saturation-mutagenesis at position TmoA I100 resulted in the finding of novel HTyr-producing variants I100A, I100S and I100G.

1. Introduction

Hydroxytyrosol (HTyr, 4-(2-(hydroxyethyl)-1,2-benzendiol)) is one of the main phenolic compounds in olives, virgin oil and waste water obtained during the production of olive oil (Briante, Patumi, Febbraio, & Nucci, 2004; Tripoli et al., 2005). HTyr has been proven to be the antioxidant with the highest free radical scavenging capacity, and is more active than antioxidant vitamins (E and C) as well as the synthetic antioxidants (Briante et al., 2004; Manna, Migliardi, Sannino, De Martino, & Capasso, 2005). *In vivo* and *in vitro* studies have shown many beneficial attributes of this *ortho*-diphenol such as anti-inflammatory activity (Tripoli et al., 2005) and inhibition of platelet aggregation (Petroni et al., 1995). Similar to other polyphenols, HTyr was shown to prevent atherosclerosis through inhibition of LDL oxidation and decrease of LDL uptake by macrophages (Ferrari, 2004; Marrugat et al., 2004; Tripoli et al., 2005). Additionally, polyphenols extracted from virgin olive oil (containing mainly HTyr), were also shown to inhibit colon carcinogenesis (Gill et al., 2005) and proliferation of human leukaemia cells (Fabiani et al., 2006). Thus, the vast amount of data accumulated regarding the benefits of HTyr, together with its high bioavailability in humans (Vissers, Zock, & Katan, 2004), make it a good candidate for potential utilisation as an antioxidant for either pharmaceutical or food preparations (i.e. functional foods (Larrosa, Espin, & Tomas-Barberan, 2003)).

Several methods have been described in the literature for the preparation of HTyr, the most common being extraction from olives or olive oil waste streams (Briante et al., 2004; Villanova, Villanova, Fasiello, & Merendino, 2006) and many of these methods have been patented (Alvin & Sniderman, 2005; Beverungen, Rull, Kempers, & Buchwald-Werner, 2005). Additionally, a chemical synthesis of HTyr has been reported, based on the reduction of 3,4-dihydroxyphenylacetic acid with $LiAlH_4$ (Capasso, Evidente, Avolio, & Solla, 1999). There are two principal biocatalytic routes for production of HTyr, both based on the conversion of tyrosol (Tyr) into HTyr via a single oxidation step. The first is based on an isolated microorganism able to grow on Tyr as a sole carbon source (Allouche, Damak, Ellouz, & Sayadi, 2004; Allouche & Sayadi, 2005). The second approach is based on the hydroxylation of Tyr by tyrosinase followed by treatment with ascorbic acid (Espin, Soler-Rivas, Cantos, Tomas-Barberan, & Wichers, 2001). Ascorbic acid is added in an equimolar amount to reduce the formed quinone back to the desired HTyr. The latter approach suffers from the need of a purification chromatographic step to remove ascorbic acid and other intermediates.

This work describes the synthesis of HTyr by the use of toluene monooxygenases (TMOs) to perform successive double hydroxylation of 2-phenylethanol (PEA), an inexpensive and abundant substrate (the price of PEA is 50 $/kg, whereas Tyr, the substrate used in previous studies, is sold for 9500 $/kg by the same supplier). The reaction scheme is depicted in Fig. 1. TMOs are soluble, non-haeme, diiron-containing enzymes belonging to a group of four component alkene/aromatic monooxygenases (Leahy, Batchelor, & Morcomb, 2003). The most extensively studied monooxygenases from this class are toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4 which hydroxylates toluene at the *ortho* position to form *o*-cresol (Canada, Iwashita, Shim, & Wood, 2002),

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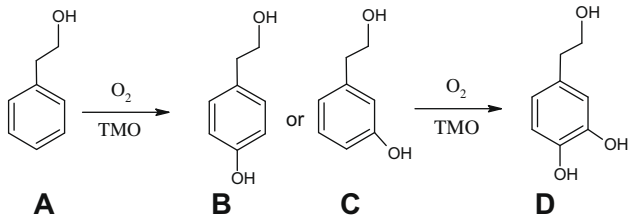

Fig. 1. Proposed synthetic route of hydroxylation of PEA (A) by TMOs to form *p*-Tyr (B) or *m*-Tyr (C) as intermediate products, and HTyr (D) as the final product.

toluene *o*/xylene monooxygenase (ToMO) of *Pseudomonas stutzeri* OX1 with a relaxed regiospecificity producing a mixture of three isomers from toluene hydroxylation (Vardar, Ryu, & Wood, 2005), toluene *p*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1 and toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1 both *para*-hydroxylating enzymes producing primarily *p*-cresol from toluene (Fishman, Tao, Vardar, Rui, & Wood, 2006; Mitchell, Rogge, Gierahn, & Fox, 2003; Tao, Fishman, Bentley, & Wood, 2004). TMOs have been shown to be effective catalysts for preparation of substituted dihydroxy-benzenes. Nitrohydroquinone (precursor for therapeutics of Parkinson's disease), 4-nitrocatechol (inhibitor of nitric oxide synthase) and 3-methoxycatechol (intermediate for an antivascular agent) are some of the compounds that were prepared using this class of enzymes (Fishman et al., 2006). Protein engineering was used to modify the regioselectivity of these enzymes through mutations at the hydroxylase gene (Fishman, Tao, Rui, & Wood, 2005; McClay, Boss, Keresztes, & Steffan, 2005; Pikus, Studts, McClay, Steffan, & Fox, 1997; Tao et al., 2004; Vardar et al., 2005). The crystal structure of the hydroxylase protein of ToMO was published in 2004 (Sazinsky, Bard, Di Donato, & Lippard, 2004), and extensive biochemical information including reaction kinetics and fundamental properties of T4MO were described by the Fox group in recent years (Bailey, McCoy, Phillips, & Fox, 2008).

The goal of this work was to develop a novel "green" route for the synthesis of HTyr using TMOs as biocatalysts, as well as to enhance the understanding of structure–function relationships of these enzymes. Both saturation-mutagenesis at known "hot spots" and directed evolution approaches were used to design enzyme variants with the desired activity and selectivity.

2. Materials and methods

2.1. Chemicals

2-Phenylethanol (PEA), *o*-, *m*-, *p*-tyrosol (Tyr), ethyl benzene (EB), benzyl alcohol (BA), 4-ethyl phenol (EP), 4-hydroxybenzyl alcohol (HBA), 4-propyl phenol (PP) and *N*,*O*-bis(trimethylsilyl)-acetamide were purchased from Sigma–Aldrich Chemical Co. (Sigma–Aldrich, Rehovot, Israel). *n*-Propyl benzene (PB) was obtained from Acros organics (Geel, Belgium). Ethyl catechol (EC) was purchased from Alfa Aesar (MA, USA) and 3,4-dihydroxybenzyl alcohol (DHBA) was purchased from TCI (Tokyo, Japan). Hydroxytyrosol (HTyr) was obtained from Cayman chemical Co. (MI, USA). All standards were prepared as stock solutions in ethanol. All materials used were of the highest purity available and were used without further purification.

2.2. Bacterial strains and growth conditions

Escherichia coli TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F' [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) with the plasmid constructs was routinely cultivated at 37 °C with shaking at 250 rpm on a TU-400 incubator shaker (Orbital shaker incubator, MRC, Holon, Israel) in Luria-Bertani (LB) medium (Sambrook & Russell, 2001) supplemented with kanamycin at 100 μg/ml to maintain the plasmids. To stably and constitutively express the toluene monooxygenase genes from the same promoter, the expression vectors pBS(Kan)TOM (henceforth TOM), pBS(Kan)TpMO (henceforth TpMO), pBS(Kan)ToMO (henceforth ToMO), and pBS(Kan)T4MO (henceforth T4MO), were constructed as described earlier (Feingersch, Shainsky, Wood, & Fishman, 2008). All experiments were conducted by diluting overnight cells to an optical density (OD) at 600 nm of 0.1 and growing to an OD of 1.3. The exponentially-grown cells were centrifuged at 8000 g for 10 min at 25 °C in a Sigma-4K15 centrifuge (Sigma, Osterode, Germany) and re-suspended in potassium phosphate buffer (100 mM, pH 7.0).

2.3. Protein analysis and molecular techniques

Protein samples of cells grown with and without 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) were analyzed on standard 12% Lammeli discontinuous sodium dodecyl sulfate (SDS)–polyacrylamide gels (Sambrook & Russell, 2001).

Plasmid DNA was isolated using a Midi Kit (Promega, WI, USA) or Mini Kit (Qiagen, CA, USA), and DNA fragments were isolated from agarose gels using the RBC extraction kit (Real Biotech Corp., Taipei, Taiwan) or collected using the E-gel® CloneWell™ system comprised of 0.8% SYBR Safe™ gels, and E-gel® iBaseTM as the electrophoresis unit (Invitrogen, Carlsbad, California). Transformation of plasmids into *E. coli* cells was performed via electroporation using a Micro-Pulser instrument (Bio-Rad, CA, USA) with the program Ec1 (1.8 kV, 1 pulse for a 0.1 cm cuvette).

3.1. Random mutagenesis (directed evolution) of T4MO

The *tmoAB* genes, α- and γ-hydroxylase subunits, respectively, and 20% of *tmoC* gene (1936 bp) in pBS(Kan)T4MO were amplified using error-prone PCR (epPCR). A 100 μL reaction contained 67 mM Tris–HCl (pH 8.8 at 25 °C), 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 5 mM $MgCl_2$, 0.35 mM $MnCl_2$, 1 M Betaine, 40 ng of template DNA pBS(Kan)T4MO, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 5U Taq DNA polymerase (BioTaq™, Bioline, London, UK) and 30 pmole of each primer (T4MObefEcoRI Front and T4MOAB-Rear, Table S1). The T4MObefEcoRI Front primer is upstream of the *Eco*RI restriction site, located in the multiple cloning site, and T4MOABRear primer is downstream of the naturally occurring *Aat*II site within the *tmoC* gene. A PCR program of 30 cycles of 94 °C for 1 min, 59.3 °C for 1 min, and 72 °C for 2.5 min, with a final extension of 72 °C for 7 min, was used in a in an Apollo ATC401 thermocycler (CLP, San Diego, USA). The resulting randomised PCR product was cloned into pBS(Kan)T4MO after double digestion with *Aat*II and *Eco*RI (New England Biolabs, MA, USA), replacing the corresponding fragment in the original plasmid. The resulting plasmid library was transformed into *E. coli* TG1 competent cells via electroporation.

3.2. Saturation mutagenesis

A gene library encoding all possible amino acids at position 100 of T4MO *tmoA* in pBS(Kan)T4MO and the analogous position 106 of TOM *tomA3* in pBS(Kan)TOM was constructed as described previously (Feingersch et al., 2008). The primers used for cloning are specified in Table S1.

3.3. DNA sequencing

DNA sequencing was performed using the dideoxy chain termination technique (Multidisciplinary laboratories, Technion, Haifa,

Israel). The primers T4MObefEcoRI Front, T4MO Seq1, T4MO Seq2 and T4MO Seq3 (Table S1) were used to determined the nucleotide sequence of the random mutagenesis T4MO mutants (sequencing a total of 2 kb including the *tmoAB* genes and 20% of *tmoC* gene). For determining the sequence of saturation mutagenesis mutants of T4MO I100, only the T4_100_Check primer was used and for sequencing TOM V106 mutants, primer TOM1 (Table S1) was used. Analysis of DNA sequences was done with the Vector NTI program (Invitrogen, CA, USA).

3.4. Screening method for HTyr production

The screening method is based on the instability of the reaction products. At neutral pH, the catechol derivatives, formed from the relevant substrate, auto-oxidise to quinones and semiquinones that readily polymerise and form a red or orange colour. The screening method used to identify mutants with a different or improved activity was described previously (Fishman, Tao, Bentley, & Wood, 2004). Mutant libraries were screened on PEA, *o*-Tyr, *m*-Tyr and *p*-Tyr, and clones showing red halo on the agar plates were further examined by GC/MS analysis.

3.5. Whole-cell enzymatic biotransformations

Whole-cell activity assays were performed in a similar manner to previous studies with TMOs (Feingersch et al., 2008; Fishman et al., 2004). Shortly, exponential-phase cultures were used in all experiments by diluting overnight cell cultures to an OD (600 nm) of 0.1 and growing to an OD of ~1.3. The exponentially-grown TG1 cells harboring the various pBS(Kan) vectors, were centrifuged at 8000 g for 10 min at 25 °C in a Sigma-4K15 centrifuge, and re-suspended in phosphate buffer to an OD of 11. The biotransformation was carried out in 16-ml glass vials containing 2 ml cells and 0.25 mM substrate (added from a 100 mM stock solution in ethanol). The vials were shaken at 600 rpm (Vibramax 100, Heidolph, Nurenberg, Germany) at room temperature. Among the substrates studied in this work, EB and PB follow Henry's law (H = 0.32 or 0.28, respectively (units less)) (Yaffe, Cohen, Espinosa, Arenas, & Giralt, 2003). Accordingly, 21 ml glass vials containing the cell suspensions (2 ml) were each sealed with a Teflon-coated septum and aluminium crimp seal, and the substrates were added by injection with a syringe. The added concentration was 1.01 mM EB or 0.92 mM PB to ensure a concentration of 0.25 mM in the aqueous phase. *t*-Butyl benzene was used as an internal standard.

The reaction was stopped periodically (a vial was sacrificed) by filtration of the cells or by extraction with ethyl acetate. Filtration was used for reactions measured by HPLC, whereas extraction using 2 ml of ethyl acetate (1:1 v) was used for samples analyzed by GC/MS. The negative control used in these experiments was TG1/pBS(Kan) (a plasmid without the monooxygenase). The initial transformation rates were determined by sampling at 3–15 min intervals during the first 5 h. The specific activity (nmol/min/mg protein) was calculated as the ratio of the initial transformation rate and the total protein content. Total protein content was 0.22 [mg protein/ml/$OD_{600\,nm}$] for TOM and ToMO and 0.24 [mg protein/ml/$OD_{600\,nm}$] for T4MO and TpMO (Fishman et al., 2005; Tao et al., 2004). Activity data reported in this paper are based on at least two independent results.

3.6. Analytical methods

The progress of enzymatic hydroxylation of PEA was measured by reverse-phase HPLC, whereas the regiospecificity, in addition to structure verification, was determined by GC/MS analysis. HPLC analysis was performed with an Agilent 1100-series instrument (Agilent Technologies, CA, USA) using an Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm, Agilent Technologies, CA, USA) equipped with a photodiode array detector. The isocratic elution was performed with acidic H_2O (0.1% formic acid) and acetonitrile as the mobile phase at a flow rate of 1 ml/min. Compounds were identified by comparison of retention times and UV–visible spectra to those of the appropriate standards. Samples for GC/MS were analyzed using a GC 6890 N (Agilent Technologies, CA, USA) instrument equipped with a capillary HP-5 column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies), and an HP-5975 mass spectra detector (Agilent Technologies, CA, USA). The detection of HTyr by GC/MS required its derivatisation with silyl groups. In addition, the derivatisation enabled to distinguish between the different Tyr isomers. The silylation was performed by adding *N*,*O*-bis(trimethylsilyl)-acetamide (250 μl) to 400 μl of the organic extract. The solution was incubated for 20 min at 80 °C. Ethyl acetate and *N*,*O*-bis(trimethylsilyl)-acetamide were evaporated under N_2 flow, and the residue was re-dissolved in ethyl acetate (300 μl) prior to analysis. The helium carrier gas flow rate was maintained at 1 ml/min. Under these conditions, the different Tyr and HTyr isomers are separated. Identification was based on comparison of their mass spectra and retention times with those of reference standards (when possible) and by comparison with an MS library data (NIST mass spectral library V 2.0).

Specific analytical methods were developed for separation of each of the substrates (and respective products) used in this work. HPLC methods were developed for the detection of BA, BP, EP, PP and their respective catechols. The determination of the volatile compounds, EB and PB, and their respective phenol products, was performed by GC/MS analysis (without derivatisation).

A complete description of the analytical methods developed for each substrate used in this work is available in the Supplementary material, Tables S2 and S3.

4. Results and discussion

4.1. Oxidation of PEA by E. coli expressing wild-type TMOs

The proposed pathway for the production of commercially-valuable HTyr includes the selective oxidation of PEA by TMOs to form *p*- or *m*-tyrosol as intermediate products and HTyr as the final product (Fig. 1). Because TMOs are complex multi-component enzyme systems that require NADH, whole-cell recombinant *E. coli* strains expressing the different enzymes were used in all assays. The four wild-type (WT) TMOs (TOM, ToMO, TpMO, and T4MO) were evaluated for their activity and regiospecificity on PEA at a concentration of 0.25 mM. PEA was found to be a poor substrate for all TMOs studied and no HTyr formation from PEA was detected except for the formation of a different catechol derivate (2,3-dihydroxyphenyl ethanol) by TOM. It should be noted that since no 2,3-dihydroxyphenyl ethanol standard was present, the identity of this product was determined according to several GC/MS examinations and the relevant MS spectra (data not shown). The initial oxidation rates of PEA by WT TMOs were low (less than 0.07 nmol/min/mg protein, Table 1) compared to the natural substrate, toluene (initial rates of 1.3–4.4 nmol/min/mg protein reported by Fishman et al. (2005) and Vardar et al. (2005) using a similar method). Likewise, the regiospecificity of the WT strains on PEA differed from those observed on toluene. Fishman et al. (2005) reported a selectivity of 100% by WT TOM on the *ortho* position of toluene and 97% and 88% to the *para* position by WT T4MO and TpMO, respectively. In addition, Vardar et al. (2005) reported a relaxed regiospecificity of WT ToMO which oxidised toluene on the *ortho*, *meta*, and *para* positions (32%, 21%, 47%, respectively). Here, TOM hydroxylated PEA mainly (but not exclusively) on the *ortho* position (64%), whereas WT T4MO, WT TpMO, and WT ToMO showed preference

Table 1
Regiospecific oxidation of PEA and Tyr isomers by TG1 cells expressing WT TOM, ToMO, T4MO and TpMO.

Substrate	PEA				*o*-Tyr	*m*-Tyr	*p*-Tyr
Enzyme	Initial oxidation rate of PEA (nmol/min/mg protein)[c]	*o*-Tyr (%)	*m*-Tyr (%)	*p*-Tyr (%)	2,3-Dihydroxyphenyl ethanol (%)	2,3-Dihydroxyphenyl ethanol (%)	3,4-Dihydroxyphenyl ethanol (HTyr) (%)
TOM[a]	0.045	64	36	–	83	9	10
ToMO[a]	0.051	–	99	1	–	–	–
T4MO[b]	0.023	–	63	37	–	–	–
TpMO[b]	0.064	–	64	36	–	–	–

The initial PEA oxidation rates are based on HPLC analysis whereas regiospecificity was determined with GC/MS analysis after 24 h. The initial substrate concentrations were 0.25 mM.

[a] Based on 0.22 mg protein/ml/$OD_{600\ nm}$.

[b] Based on 0.24 mg protein/ml/$OD_{600\ nm}$.

[c] Results represent an average of at least three independent experiments, with standard deviation of less than 8%.

for the *meta* position (63%, 64% and 99%, respectively) (Table 1). Interestingly, whereas TOM, T4MO and TpMO were not highly regioselective on PEA, ToMO displayed high regioselectivity towards the *meta* position. Among the TMOs discovered to date, all were reported to perform primarily *ortho*- or *para*-hydroxylation of toluene and other substrates (Fishman et al., 2005). Considering that the PEA substituent is an *ortho* or *para* directing group (Solomons & Fryhle, 2008), the high specificity obtained by ToMO towards the *meta* position is unusual and has high potential for a straightforward selective synthesis of *m*-Tyr.

Employing the different Tyr isomers as substrates revealed that only TOM, which is considered a phenol hydroxylase (Leahy et al., 2003), was capable of oxidising the phenolic substrates to the corresponding catechols (Table 1). It was discovered that WT TOM hydroxylates both *o*-Tyr and *m*-Tyr to form 2,3-dihydroxyphenyl ethanol, with preference for the *o*-Tyr oxidation (83% conversion after 24 h compared to 9% coversion of *m*-Tyr). WT TOM was capable of forming the desirable HTyr (10% conversion after 24 h) by oxidising *p*-Tyr, which is not one of the intermediate products received from PEA by TOM. Although TOM can be a good candidate for the biosynthesis of HTyr, the high cost of the substrate (*p*-Tyr) represents the major drawback. Compared with *p*-Tyr, PEA is an inexpensive substance and consequently it is preferable as a starting material. Hence, this study focused on the biosynthesis of HTyr using PEA as the substrate.

4.2. Oxidation of PEA and structurally-related substrates by WT T4MO

For a better understanding of the factors responsible for the poor hydroxylation activity on PEA, the relationship between substrate structure and enzyme function was studied in detail using various structurally-related substrates (EB, BA, PB and their corresponding phenol derivates, Fig. 2E–G) which differ from PEA and the natural substrate, toluene, in the length of the alkyl side chain and the presence of a hydroxyl group. It is worth mentioning that EB and PB tend to evaporate from the aqueous phase and therefore the cells may experience lower actual concentrations than the ones intended. Accordingly, new conditions were established for the EB and PB biotransformations and the applied concentrations were calculated based on Henry's law. T4MO was chosen for this study based on its preferred *para*-hydroxylation of toluene and other mono substituted benzenes (Fishman et al., 2004; Pikus et al., 1997). Additionally, it was shown that T4MO expressed in *E. coli* TG1 cells can produce catechol derivatives by successive mono- and di-hydroxylations on the *meta* and *para* positions (Fishman et al., 2004; Fishman et al., 2006; Tao et al., 2004).

As shown in Fig. 3, the absence of the hydroxyl group and the shorter alkyl chain in EB result in a faster hydroxylation rate (Two orders of magnitude higher than PEA), whereas the short alkyl chain with the presence of the hydroxyl group in BA, resulted in a higher rate compared to PEA but lower than the EB hydroxylation rate (EB hydroxylation is 6-fold faster than the BA hydroxylation rate) (Fig. 3). The initial PB hydroxylation rate is similar to the one of BA, suggesting that their side residues may have the same impact on the enzymatic activity. A similar tendency was observed for oxidation of the phenol derivatives, however, all of the hydroxylation rates were lower (one order of magnitude). Additionally, T4MO was unable to oxidise *p*-Tyr and 4-HBA, both containing a hydroxyl group on the side chain. These results clearly indicate that the length of the alkyl side chain and the presence of the hydroxyl group on the side chain influence the enzymatic activity. It is assumed that the bulky and hydrophilic nature of the PEA side chain interferes with the substrates' entrance and orientation in the active site. Therefore, it was postulated that increasing the width of the entrance channel and/or the size of the active site pocket may allow higher conversion rates on PEA.

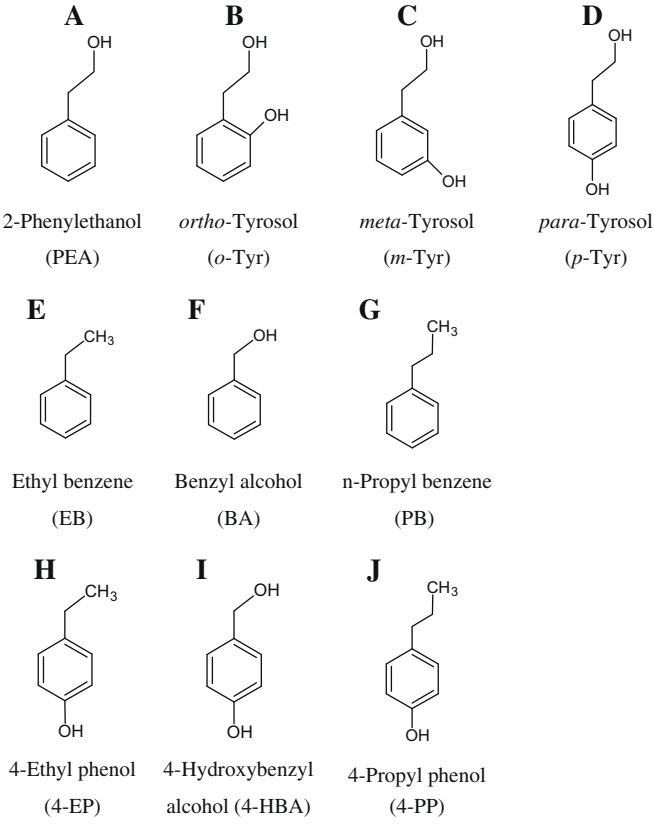

Fig. 2. Chemical structures of the various substrates used in this study. The substrates differ from one another and from the natural substrate, toluene, in the length of the alkyl side chain and the presence of a hydroxyl group.

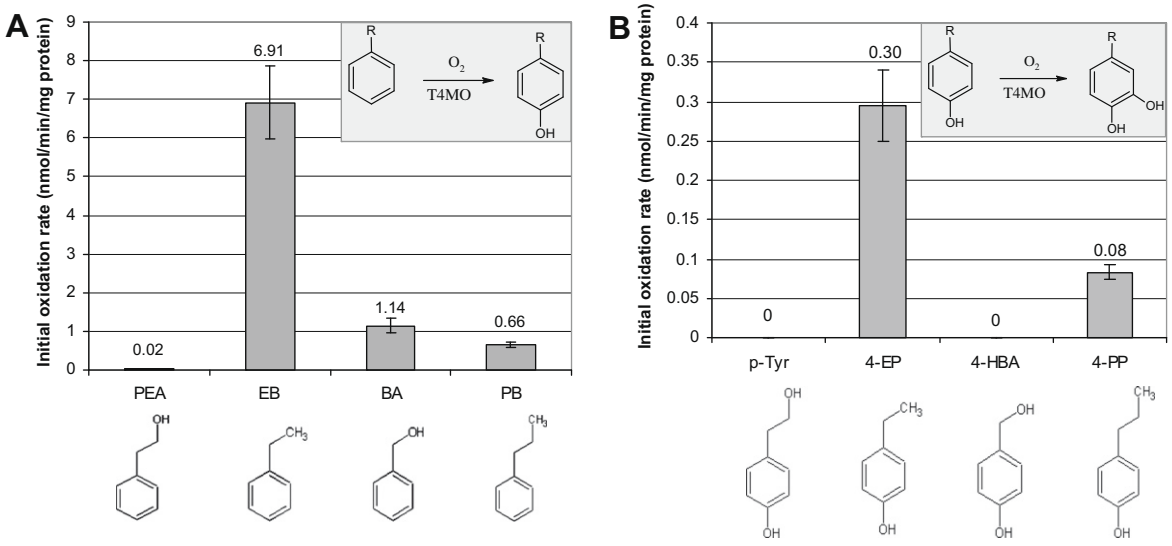

Fig. 3. Initial transformation rates of T4MO for oxidation of the structurally-related substrates: (A) PEA, EB, BA and PB; (B) *p*-Tyr, 4-EP, 4-HBA and 4-PP. Activity on PEA, PB and the phenol derivates was determined via HPLC analysis (initial substrate concentrations of 0.25 mM). Activity on EB and PB was determined with GC/MS analysis at initial substrate concentrations of 0.25 mM based on Henry's law constant of 0.32 or 0.28, respectively (Yaffe et al., 2003) (1.01 and 0.92 mM, respectively, were added as if all the substrate was in the liquid phase).

4.3. Directed evolution of the T4MO α-hydroxylase subunit

The non-rational approach of directed evolution was performed, using error-prone PCR amplification of the α-and γ-hydroxylase subunits (and ~20% of the ferredoxin subunit) of T4MO in order to change and improve its regiospecificity and activity. A library of over 3000 mutants was generated and screened on four substrates, namely, PEA, *o*-Tyr, *m*-Tyr and *p*-Tyr. A rapid nylon membrane plate assay was used to screen for the production of catechol derivatives from TG1 cells expressing T4MO variants. Several mutants with altered activity were discovered. The most active mutant (15-fold higher activity than WT T4MO, while representing a similar regiospecificity) had four silent mutations and a single amino acid change from Ser to Cys at position 395 (T4MO TmoA S395C). It was surprising to discover that the mutation is located at a new position, distant from the active site. Further examination of the S395C variant revealed that, unlike WT T4MO, it hydroxylats *o*-Tyr to form 2,3-dihydroxyphenyl ethanol (18% conversion after 44 h). In addition to the peak of 2,3-dihydroxyphenyl ethanol in the GC/MS chromatogram, another peak was observed with a similar mass spectrum. The identity of this peak is believed to be another dihydroxy-benzene product, possibly 2,5-dihydroxyphenyl ethanol. When *m*- and *p*-Tyr were used as substrates, no product peak was observed. The ability of T4MO S395C mutant to hydroxylate a Tyr isomer (*o*-Tyr), which WT T4MO is not capable of, confirms that position S395 indeed influences the activity.

4.4. Rational design

Residues T4MO TmoA I100 and TomA3 V106 in TOM, which are part of the hydrophobic cavity surrounding the diiron binding site, were shown to have a major influence on the rate and specificity of these enzymes in previous studies (Feingersch et al., 2008; Fishman et al., 2004; McClay et al., 2005; Tao, Bentley, & Wood, 2005; Tao et al., 2004). Consequently, saturation mutagenesis was performed at these positions and the activity on PEA and Tyr isomers was examined. A total of 300 variants from the T4MO TmoA I100 library were screened (as described above) to ensure with a probability of 99% that all 64 possible codons, from the single site random mutagenesis, were checked (Fishman et al., 2006). Given that WT TOM forms catechol derivatives from PEA and Tyr isomers, the screening method was not efficient for this enzyme. Therefore, only several identified mutants from the TOM TomA3 V106 library were examined. It was discovered that mutations at positions TmoA I100 in T4MO and TomA3 V106 in TOM altered both activity and regiospecificity.

The relative activity and the product distribution of the T4MO TmoA I100 variants selected from the agar plate screen on PEA, are presented in Fig. 4. The activity of TG1 cells expressing WT T4MO was improved by mutants I100A, I100S, I100D, I100 V and I100G, ranging from 21-fold for I100 V, up to 34-, 35- and 36-fold for I100S, I100G and I100A, respectively. Moreover, mutants I100A, I100S and I100G performed the di-hydroxylation of PEA to form the desirable HTyr. Another small peak (representing negligible amounts of product) was detected in the GC/MS chromatograms of variants I100A, I100S and I100G oxidising PEA or *m*-Tyr. The identity of this peak is believed to be 3,5-dihydroxyphenyl ethanol, based on its similarity with HTyr. Unlike WT T4MO, variants I100A, I100S, I100D, I100V and I100G hydroxylated *m*-Tyr to form HTyr with conversion of 55%, 50%, 2%, 3% and 18%, respectively, whereas only I100A, I100S and I100G hydroxylated *p*-Tyr to form HTyr (65%, 48% and 98% conversion, respectively) and *o*-Tyr to form 2,3-dihydroxyphenyl ethanol (24%, 11% and 13%, respectively) (Fig. S1 in Supplementary material). These results support the proposed pathway for the oxidation of PEA to HTyr through *m*- and *p*-Tyr (Fig. 1). The formation of HTyr by variants I100A and I100S is believed to be through the oxidation of both *m*- and *p*-Tyr equally. In contrast, I100G, which oxidised nearly 100% *p*-Tyr, is thought to produce HTyr primarily through the oxidation of *p*-Tyr (Fig. S1). Although variants I100D and I100 V were capable of oxidising *m*-Tyr, the conversion rates were low. In addition, minor amounts of HTyr and 2,3-dihydroxyphenyl ethanol were observed during the oxidation of *p*-Tyr and *o*-Tyr, respectively, by I100D. Subsequently, it is concluded that I100D and I100 V form HTyr from PEA but in negligible amounts.

Mutagenesis at position TomA3 V106 in TOM, the analogous position of T4MO TmoA I100, resulted in a change in both regiospecificity and activity of the TOM mutants (Fig. 5, Fig. S2). As shown in Fig. 5, the relative activity of V106S, V106A, V106L, V106 M and V106E was higher than that of the WT TOM. Increased activities

of 25- and 28-fold were observed for V106A and V106S, respectively, and 39-fold for V106E. Although WT TOM has a preference for the *ortho*-hydroxylation of PEA (Table 1), surprisingly, no *ortho* isomers were detected for the V106 variants examined. The variants were able to hydroxylate the *para* position on the benzene ring, which was not accessible by the WT TOM. Furthermore, position V106 altered the regiospecificity of the second hydroxylation step and enabled the formation of HTyr from PEA by V106S, V106A and V106E mutants (42%, 11% and 21% of HTyr obtained, respectively). Further examination revealed that altering the Val residue to Ser, Ala or Glu influenced the regiospecificity of the *m*-Tyr hydroxylation and lead to the formation of HTyr in addition to 2,3-dihydroxyphenyl ethanol (Fig. S2). These mutants formed HTyr from *m*-Tyr with regiospecificity of 90%, 15% and 62%, respectively. The 83% conversion of *o*-Tyr to 2,3-dihydroxyphenyl ethanol by WT TOM was improved to 93% by V106 M, 97% by V106S, 99% by V106L and V106E and up to full conversion (100%) by V106A. The conversion of *p*-Tyr to HTyr (10% by WT TOM) was enhanced by mutants V106S, V106A and V106E (14%, 17% and 22%, respectively) whereas mutants V106L and V106 M formed negligible amounts of HTyr.

It was previously shown by others (Canada et al., 2002; Feingersch et al., 2008; Fishman et al., 2004; Tao et al., 2004; Tao et al., 2005) that residue I100 of T4MO TmoA and the analogous residue V106 in TOM TomA3 have a major influence on the rate and specificity of these enzymes. For instance, it was reported that T4MO I100S and I100A variants had higher oxidation rates on toluene, nitrobenzene and nitrophenol (Fishman et al., 2004) and TOM V106A variant was able to oxidise bulky polyaromatics (as phenanthrene) at higher rates (Canada et al., 2002). These residues have been proposed to act as a gate which controls substrate and product transport to the diiron active centre. This assumption is further supported by our results regarding both T4MO I100 and TOM V106 variants and explains the improved activity observed on PEA – a mono-aromatic substance with a bulky and hydrophilic side chain.

It should be noted that the hydrophilic nature of HTyr containing three hydroxyl groups (one on the side chain and two on the aromatic ring) impairs its extraction from the aqueous biotransformation medium. Moreover, since HTyr is a catechol derivative, it may undergo rapid auto-oxidation during the incubation period. HPLC and GC/MS analyses strengthen this assumption and reveal that approximately 15% of the starting HTyr concentration is decomposed during the first 24 h and another 10% remain within the aqueous phase after the extraction (results not shown). Consequently, the actual quantities of HTyr formed during the biotrasformations are higher than the amounts detected. It is expected that further optimisation of the process as well as biochemical engineering approaches, as fed-batch or continuous feeding, cell immobilisation and product adsorption on solid supports, will lead to an improved yield and productivity.

The fact that the negative control, *E. coli* TG1/pBS(Kan), did not produce any products from the substrates tested and did not degrade any of the products formed in all the biotransformations

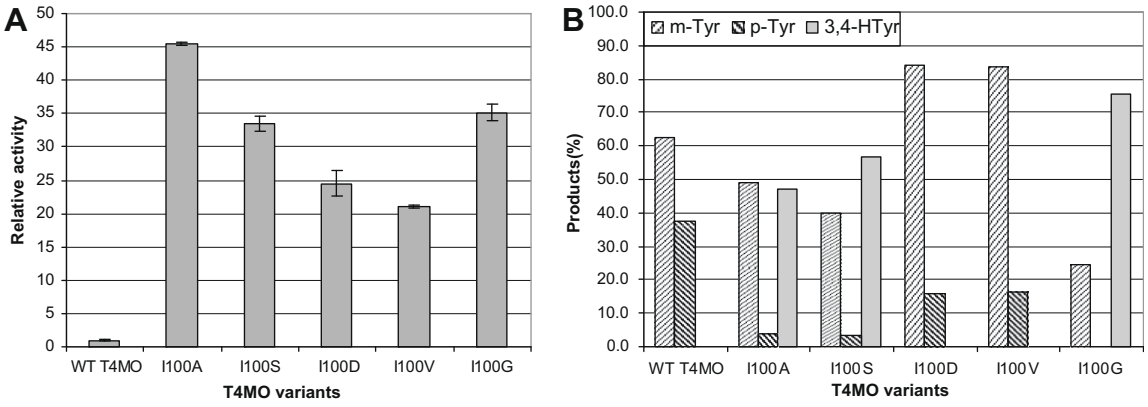

Fig. 4. Relative activity on PEA (A) and product distribution (B) of T4MO I100 variants. The relative activity is normalised to WT T4MO designated as 1. The activity and regiospecificity were determined via GC/MS analysis over a 24 h time period, with initial PEA concentration of 0.25 mM.

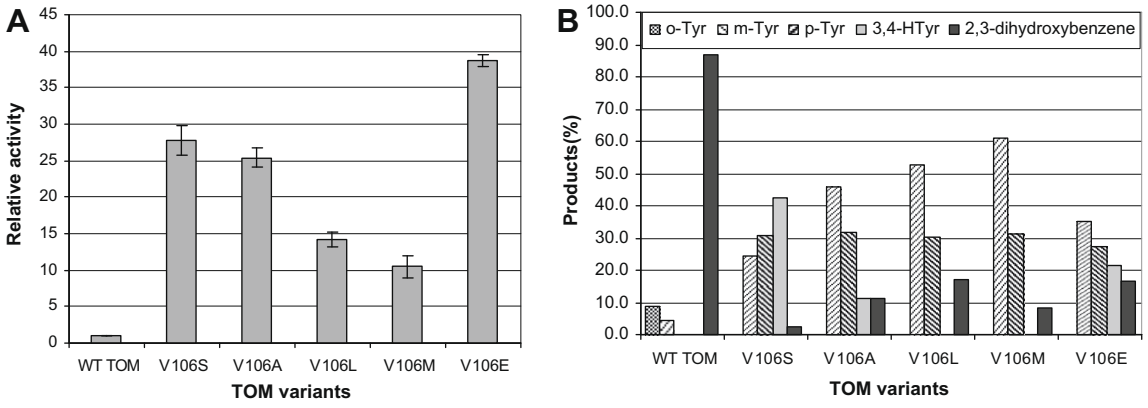

Fig. 5. Relative activity (A) and regiospecificity (B) on PEA of TOM V106 variants. This position was chosen based on previous studies with other substrates. The activity is normalised to WT TOM designated as 1. Reaction conditions were as described in the legend of Fig. 4.

examined, indicates that the products were formed due to hydroxylation by the cloned TMOs. To verify that the increase in activity of the mutants is derived from the amino acid substitutions rather than expression level changes, the TmoA (55 kDa), TmoE (35 kDa) and TmoF (36 kDa) subunits in T4MO, and the analogous TomA3 (61 kD), TomA1 (37 kD) and TomA5 (39 kD) subunits in TOM were visualised on gel by using SDS–PAGE for the mutants and WT enzymes. All of the mutant and WT bands had similar intensities. As the cell growth and the biotransformation conditions were identical for the WT and mutants, the changes in activity appear to arise from the mutations at TmoA I100 for T4MO or TomA3 V106 for TOM and not from different expression levels.

5. Conclusions

This work describes a novel method for the synthesis of HTyr with TMOs via two successive hydroxylations of PEA. TMOs are capable of oxidising a large spectrum of substrates such as substituted aromatic and phenolic compounds. Despite the resemblance of PEA to the natural substrate toluene, it was found to be a poor substrate for all enzymes studied. By investigating structure–function correlations, it was discovered that the increase in length of the alkyl side chain as well as the presence of the hydroxyl group influence the activity in an additive manner, possibly by interfering with the substrates' entrance into the active site. Using rational and random protein engineering approaches, the activity of T4MO and TOM enzymes were further improved and led to the discovery of a new important position, T4MO TmoA S395. This position, distant from the active site, was not previously reported in the literature and is being further investigated in our lab. By generating mutations within the active side pocket at position I100 of T4MO, it was discovered that increasing the size of the pocket enables HTyr formation, which WT T4MO is not capable of. The structure–function lessons learned from the oxidation of various mono- and di-substituted aromatic substrates, as well as from the mutagenesis experiments may be applicable for a broad range of other bulky substrates. Moreover, this is the first evidence of HTyr formation from PEA by any biocatalyst. The process is currently being optimised for higher yields and for other beneficial mutations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.02.020.

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