Biobased epoxides from unsaturated plant fatty acids by a new fungal peroxygenase and its heme-channel variants

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ABSTRACT

Enzyme-driven oxygenation reactions are in the spotlight for organic synthesis, and production of reactive bio-based compounds. The oxirane ring of epoxides has been termed the "Lord of chemical rings" because of its high reactivity and interest in chemical syntheses. A heme-thiolate unspecific peroxygenase (UPO) from the fungus *Collariella virescens* has recently proven to be a suitable catalyst for the selective production of fatty acid (di)epoxides. Here, lipid epoxidation is investigated in the frame of the SusBind EU project (https://susbind.eu) whose objective is the production of sustainable binders for wood-based panels.

Keywords: *Vegetable oils, enzymatic oxyfunctionalization, fatty-acid epoxides, unspecific peroxygenases (UPOs), Collariella virescens UPO, enzyme engineering, binder ingredients*

INTRODUCTION

The oxirane ring of epoxides has been termed the "Lord of the chemical rings" [1] because of its high reactivity for the industrial production of bio-based chemicals, including binder ingredients and resins. Epoxy resins and curing agents usually contain more than one reaction site per molecule, to allow multiple crosslink reactions between them. Vegetable oils are important renewable feedstocks for a bio-based chemical industry [2]. The epoxides produced from oil fatty acids can be renewable ingredients for epoxy resins, as long as they meet the required reaction selectivity and cross-linking properties.

Lipid epoxidation is industrially performed by the Prileschajew reaction [3] *via* percarboxylic acids generated by strong acids. Attempts to use milder conditions include lipase-H_2O_2 reactions [4], which maintain the drawbacks due to the use of peracids, and direct enzymatic epoxidation. Some plant peroxygenases [5], P450 monooxygenases [6], and fungal unspecific peroxygenases (UPOs) [7] catalyze the direct epoxidation of (poly)unsaturated fatty acids. The latter enzymes present advantages related to their self-sufficiency, stability and secreted nature [8]. UPOs were known as aromatic peroxygenases [9] but numerous examples have shown their wide versatility on aliphatic compounds including epoxidation reactions [7].

However, although the presence of over 8,000 UPO-type genes in sequenced fungal genomes (https://mycocosm.jgi.doe.gov) has been recently reported [8], only a handful UPO proteins are yet available, and UPO crystal structures from only four fungal species have been published to date [10-12]. Among them, *Collariella virescens* UPO is only known as a

recombinant protein (r*Cvi*UPO) heterologously expressed in *Escherichia coli* as an active soluble enzyme [13]. The obtained amounts are suitable for structure-function and (analytical scale) reaction optimization and, together with its available crystal structure [10], make r*Cvi*UPO a good starting point for future studies. Interestingly, r*Cvi*UPO shows good conversion of unsaturated fatty acids and, in contrast to other UPOs, generates epoxides as main products [14].

EXPERIMENTAL

r*Cvi*UPO was produced as a cytosolic active enzyme in *E. coli* and purified as previously described [13]. Its solved crystal structure (PDB entry 7ZCL) was used for the design of four mutated variants with progressively enlarged heme access channel (**Fig. 1**). Single and double mutations were introduced in the *Cvi*UPO gene (cloned in pET23a) using the Expand Long Template PCR kit, and the corresponding mutagenic primers, while the gene of a sextuple variant was synthesized as reported for the wild C*vi*UPO gene [13]. All the variants were produced as reported for the native (non-mutated recombinant) enzyme.

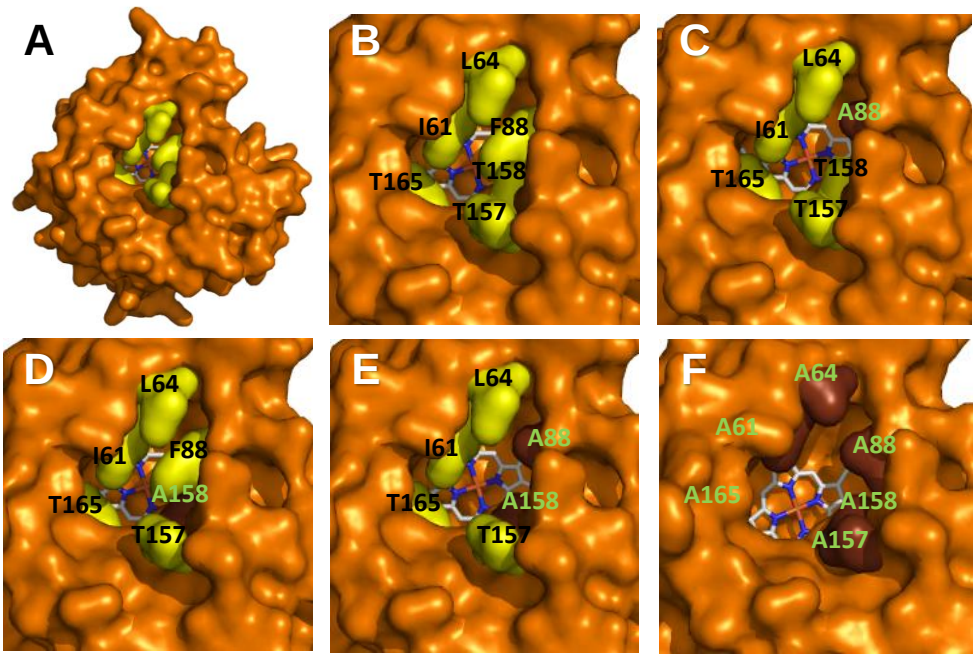

Figure 1. Solvent-access surface in *Cvi*UPO (**A**), and detail of the to the heme (CPK sticks) access channel in the native enzyme (**B**) and its F88A (**C**), T158A (**D**), F88A/T158A (**E**) and 6Ala (**F**) variants. Residues to be mutated (black labels) have yellow surfaces and the introduced alanines (yellow labels) show brown surfaces.

For evaluating its UPO epoxidation ability, 0.1 mM oleic (C18:1), linoleic (C18:2) and α-linolenic (C18:3) acids were used as r*Cvi*UPO substrates, in 30 min reactions containing 1.25 mM H_2O_2 in 50 mM phosphate (pH 7) and 20% acetone. Sunflower oil hydrolyzate was also assayed for epoxide production after saponification and extraction of the hydrolyzed fatty acids. Initial reactions were performed under the same conditions used with individual fatty acids. But, up to 10 mM hydrolyzate was used in further reactions and the effect of several variables was studied. Given the results obtained with isolated fatty acids, the F88A/T158A variant was selected for the hydrolyzate reactions.

Reaction products were liquid-liquid extracted with methyl *tert*-butyl ether, and analyzed by GC-MS as TMSi derivatives. Epoxidation yields were calculated taking into account the epoxidation degree, number of unsaturations and reaction conversion for each substrate.

RESULTS AND DISCUSSION

Previous results with r*Cvi*UPO variants [14] led us to study further broadening of the heme channel. For simplicity, the residues surrounding the channel opening were mutated into alanines; and two simple (F88A and T158A), one double (F88A/T158A) and one sextuple (I61A/L64A/F88A/T157A/T158A/T165A) variants were designed (**Fig. 1**).

GC-MS analyses revealed different oxygenation products in the reactions of isolated 18-C unsaturated fatty acids with r*Cvi*UPO and the above variants. Among the different oxygenated products identified (**Table 1**), the most interesting differences were observed in the formation of C18:1/C18:2 diepoxides. These compounds, of interest as cross-linking molecules, were absent from the r*Cvi*UPO reactions but amounted >80% in the double-variant reactions.

Table 1. Fatty acid conversion and percentages of main products by r*Cvi*UPO and its heme-channel variants.

	Conversion	Products (%)					
	(%)	15-Epoxy	12-Epoxy	9-Epoxy	Diepoxy	OH/keto	OH-Epoxy
C18:1							
r*Cvi*UPO	96	-	-	71	-	28	1
F88A	97	-	-	69	-	6	25
T158A	98	-	-	87	-	5	8
F88A/T158A	95	-	-	63	-	13	24
6Ala	96	-	-	96	-	4	-
C18:2							
r*Cvi*UPO	97	-	56	10	-	8	26
F88A	98	-	15	2	46	12	25
T158A	88	-	23	17	29	27	4
F88A/T158A	99	-	4	-	81	-	15
6Ala	99	-	-	25	64	-	11
C18:3							
r*Cvi*UPO	96	77	6	2	-	-	15
F88A	98	16	6	4	53	8	13
T158A	98	26	30	17	3	20	3
F88A/T158A	99	2	3	-	82	-	13
6Ala	99	17	35	16	10	16	6

Under the same conditions used for individual fatty acids, the double variant also enabled epoxidation of hydrolyzed sunflower oil with epoxidation yield (referred to the total of double bonds present) higher than obtained with the native enzyme. Moreover, optimized conditions (using 50 M enzyme, 30% acetone, 25 mM H_2O_2 and 60-min reaction time) permitted to increase (x100) the hydrolyzate concentration maintaining epoxidation yields of 85% after 1 h reaction with the mutated double variant.

CONCLUSIONS

The results presented illustrate how *E. coli* expression can speed up the availability of new UPOs, and the design of *ad hoc* variants. These self-sufficient mono(per)oxygenases have been termed "dream biocatalysts" for some selective oxyfunctionalization reactions that are difficult (often nearly impossible) to be completed only by chemical means [15].

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