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COMMUNICATION

Engineering Fatty Acid Photodecarboxylase To Enable the Highly Selective Decarboxylation of *trans* Fatty Acids

Danyang Li, Tao Han, Jiadan Xue, Weihua Xu, Jian Xu*, Qi Wu*[a]

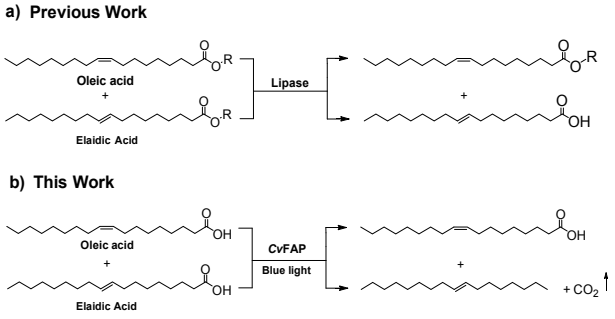
Abstract: Due to the high risk of heart disease caused by the intake of *trans* fatty acids, a method to eliminate *trans* fatty acids from foods has become a critical issue. Herein, we engineered fatty acid photodecarboxylase from *Chlorella variabilis* (*Cv*FAP) to selectively catalyze the decarboxylation of *trans* fatty acids to yield readily-removed hydrocarbons and carbon dioxide, while *cis* fatty acids remained unchanged. An efficient protein engineering based on FRISM strategy was implemented to intensify the electronic interaction between the residues and the double bond of the substrate that stabilized the binding of elaidic acid in the channel. For the model compounds, oleic acid and elaidic acid, the best mutant, V453E, showed a one-thousand-fold improvement in the *trans-over-cis* (*ToC*) selectivity compared with wild type (WT). As the first report of the direct biocatalytic decarboxylation resolution of *trans*/*cis* fatty acids, this work offers a safe, facile, and eco-friendly process to eliminate *trans* fatty acids from edible oils.

Vegetable oil, which mainly consists of saturated fatty acids (palmitic and stearic) and unsaturated fatty acids (oleic, linoleic, and linolenic), is considered an important source of energy for humans and structural components for cells. However, previous studies have shown that excessive intake of saturated fatty acids increases the risk of cardiovascular diseases.[1] In contrast to saturated fatty acids, unsaturated fatty acids are healthier, and can protect against cardiac arrhythmia and may reduce blood pressure.[2] In-depth food science studies have shown that *trans* fatty acids are undesired components of unsaturated fatty acids, which have some significant side effects on humans, including inducing heart disease or metabolic dysfunction.[2-3] As a consequence, on January 1, 2006, the Food and Drug Administration (FDA) ruled that the nutrition labels for all conventional foods and supplements must indicate the content of *trans* fatty acids.[4] Thus, the construction of protocols to separate *cis* fatty acids from *trans* fatty acids, and even saturated fatty acids represents a practically important food safety problem, as well as a fundamentally important research topic.

Due to its green, environmentally-friendly, and non-toxic nature, biocatalysis is an attractive technology in food processing industries.[5] Unfortunately, few methods have been reported for selectively eliminating *trans* fatty acids from mixtures of different kinds of fatty acids. Among them, Warwel's group assessed the selectivity of oleic and elaidic acid by esterification with a series of lipases.[6] Bornscheuer and co-workers reported an engineered lipase with a high selectivity for the *p*-nitrophenol ester of *trans* fatty acids.[7] An important limitation of these methods is the additional esterification step, which complicates the reaction system; thus, it is challenging to develop a direct biocatalytic method to rapidly and efficiently eliminate *trans* fatty acids from a mixture.

Recently, Bession and co-workers reported a fatty acid photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP), and then used it to convert long-chain fatty acids into hydrocarbons.[8] The novel catalytic activity relied on the irradiation of flavin adenine dinucleotide (FAD) by visible light, which induced decarboxylation through a radical-based mechanism.[8] Then, Hollmann and co-workers successfully developed a series of cascade reactions by employing *Cv*FAP and other enzymes to produce valuable compounds.[9] With the efforts of directed evolution, Scrutton's group and our group have evolved the catalytic activity of *Cv*FAP for diverse substrates.[10] These works have provided further insight into the catalytic mechanism of *Cv*FAP and expanded the application scope of this enzyme. Thus, considering the significant structural difference between *cis* and *trans* fatty acids, we speculated that the specific structure of the engineered *Cv*FAP could provide a potential method to separate *cis* and *trans* fatty acids.

Herein, we report, to the best of our knowledge, the first example of the selective elimination of *trans* fatty acids by direct enzymatic decarboxylation (Scheme 1). This biotransformation proceeds by harnessing visible light as a safe, inexpensive, and clean source of energy, which provides great potential in the food safety field.



Scheme 1. The selective reaction of *trans/cis* fatty acids catalyzed by enzymes.

We chose oleic acid and elaidic acid as model substrates due to their similar physical properties and their frequent occurrence in vegetable oils.[1] Initially, a problem in the enzymatic process is the low aqueous solubility of the substrate. However, the introduction of organic solvents to dissolve the substrate may greatly affect the catalytic performance of enzymes or even denature them.[11] Thus, choosing a proper co-solvent is the first step. As shown in Figure 1, WT-*Cv*FAP displayed poor or even

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reversed *trans*-over-*cis* (*ToC*) selectivity in all of the tested solvents. Due to both the relatively high activity and *ToC* selectivity observed in methanol, this solvent was chosen for subsequent mutagenesis and screening.

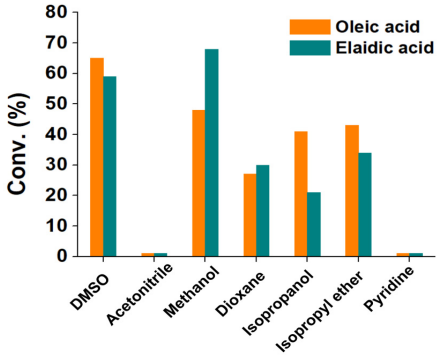

Figure 1. The co-solvent screening of a model reaction catalyzed by WT-*Cv*FAP. Oleic acid or elaidic acid dissolved in different solvents (100 µL, 70 mM) was added to 900 µL cell extract of WT-*Cv*FAP (Tris-HCl buffer 50 mM, pH 8.5). The reaction mixture was shaken at 30 °C under irradiation for 0.5 h.

It is well known that high-throughput screening processes are expensive and have large workloads, which have become the bottlenecks of directed evolution.[12] In order to improve the evolution efficiency, we recently developed a novel strategy, dubbed "focused rational iterative site-specific mutagenesis" (FRISM), [13] which calls for the rational selection of a highly limited set of amino acids to be introduced at the chosen mutation sites by site-specific mutagenesis in an iterative manner. The identification of appropriate mutational residues (hotspots) is essential in this strategy. By means of molecular docking, a reduced number of residues closest to the substrate are then chosen as hotspots. The next crucial phase of FRISM concerns the choice of amino acids to be introduced at the hotspots. In contrast to CAST/ISM, only 3-5 amino acids with significant characteristics, such as different steric, electrical, hydrophilic and hydrophobic properties, are suggested for each individual hotspot.[13] Based on this strategy, we first docked oleic acid and elaidic acid into the active site of the *Cv*FAP crystal structure (PDB ID: 5NCC)[8]. As shown in Figure 2, due to the restriction of the enzyme's narrow binding channel, the substrate-binding positions of the *cis-trans* isomers are similar, which are mainly stabilized by hydrophobic interactions. The location of the Δ9 double bond aroused our attention due to the significant difference of orientation and relatively rigid structure compared with other alkane chains. We speculated that modifying the binding tunnel around the Δ9 double bond might significantly affect the selectivity of *cis-trans*-isomers. Therefore, typical residues in a 5 Å zone around the Δ9 double bond, which was oriented to the binding tunnel, were chosen as key sites for the FRISM process (Figure S1). Considering the small size of the Δ9 double bond, the steric properties of the binding pocket may not be the critical factor that influences the positions of *cis-trans*-isomers according to docking. Thus, we aimed to modify the key sites with a series of electron-rich amino acids, including Phe, Glu, Asp, and Ser, to induce the formation of probable p-π or π-π stacking interactions between the Δ9 double bond and residues to further separate the two isomers.

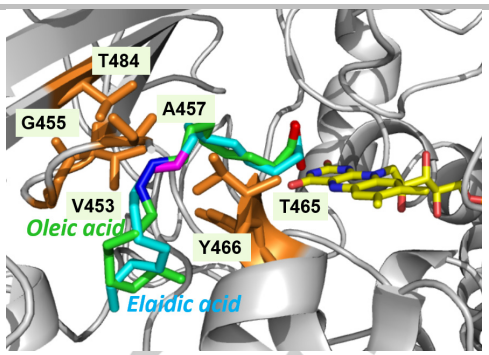

Figure 2. The crystal structure of WT-*Cv*FAP docked with oleic acid and elaidic acid. The six residues (orange) were chosen for mutagenesis by FRISM; the green stick model depicts oleic acid (Δ9 double: blue), and cyan depicts elaidic acid (Δ9 double: pink).

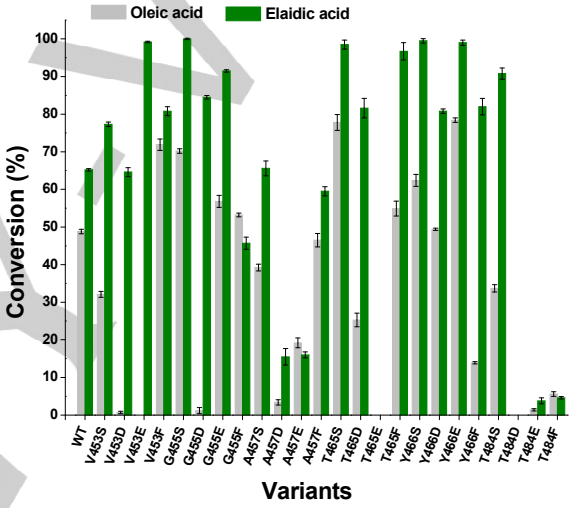

Figure 3. Assessing the introduction of electron-rich amino acids (F, E, D, S) at the sites chosen by FRISM.

The highly-focused library at the selected positions (T484, Y466, T465, A457 G455, and V453) was then screened with oleic acid and elaidic acid, respectively, and the results are shown in Figure 3. V453 and G455 displayed a clear effect on the *cis-trans* selectivity. When G455D and V453D were introduced into the binding tunnel, a more than tenfold improvement in the *cis-trans* selectivity was obtained (*ToC* = $Conv._{tans}/Conv._{cis}$, *ToC* of WT = 1.3, *ToC* of G455D = 70.4, and *ToC* of V453D = 92.3, Table S2). The most promising result was obtained by V453E, which displayed a nearly exclusive preference for elaidic acid with >99% conversion, while no decarboxylated product of oleic acid was detected by GC (*ToC* > 1000). To our delight, the best mutant occurred in the first generation of mutations, and an iterative process was not needed. The properties of the best variant were then assessed and summarized in Table S2-S4. Kinetic analysis and TON of V453E were tested, and it was found that the catalytic activity of oleic acid declined greatly compared with WT-*Cv*FAP, while the activity of elaidic acid displayed by V453E was 2.1-fold higher than that of WT (k_{cat}: 0.97 *vs.* 0.45 s^{-1}, Table S3), resulting in a high preference for elaidic acid. The thermostability of V453E was also evaluated by the T_{50} value, and the results showed that

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the mutation did not impair the thermostability of the enzyme to a significant extent.

Having the optimal mutant in hand, we then evaluated other natural fatty acids. The V453E mutant also showed a high *ToC* selectivity for several pairs of typical *trans*/*cis* fatty acids, such as vaccenic acid *vs*. *cis*-vaccenic acid, linoeladidic acid *vs.* linoleic acid, with a *ToC* value of 7.5 and 170, respectively (Table 1, entry 4 *vs.* 3, 6 *vs.* 5). In sharp contrast, WT almost displayed no selectivity, with *ToC* values≈1. Stearic acid, another undesirable saturated fatty acid, also can be consumed by the V453E mutant in satisfactory yield, probably due to its similar structure to elaidic acid.

Table 1: Assessing the reactivity of different fatty acids catalyzed by WT-CvFAP and V453E.[a]

Entry	Substrate	WT-CvFAP		V453E	
		Yield (%)	TON	Yield (%)	TON
1	Oleic acid (C18:1, Δ9 cis)	49	1162	<1	<1
2	Elaidic acid (C18:1, Δ9 trans)	65	1559	99	1080
3	Cis-vaccenic acid (C18:1, Δ11 cis)	51	1226	8	91
4	Vaccenic acid (C18:1, Δ11 trans)	37	881	61	678
5	Linoleic acid (C18:2, Δ9,12 all cis)	32	774	<1	<1
6	Linoeladidic acid (C18:2, Δ9,12 all trans)	34	810	13	170
7	Linolenic acid (C18:3, Δ9,12,15 all cis)	7.2	174	<1	<1
8	Stearic acid (C18:0)	44	1036	83	939

[a] Reaction conditions: Substrates in methanol (100 μL, 70 mM) were added to the crude cell extract (Tris-HCl buffer 50 mM, pH 8.5, 900 μL). The reaction mixture was shaken at 30 °C under irradiation for 0.5 h.

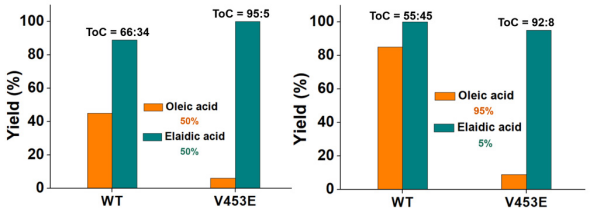

Figure 4. Assessing the selectivity and reaction yields of WT *Cv*FAP and V453E mutant in different mixtures of oleic acid and elaidic acid.

Next, a mixture of oleic acid and elaidic acid was also tested by V453E, as shown in Figure 4. The 50: 50 mixture gave a satisfactory result (99% conversion of elaidic acid with over 95:5 *ToC*). In a relatively extreme concentration of oleic acid (*cis*: *trans*= 95:5), the reaction also proceeded smoothly to provide 97% conversion of elaidic acid, with over 92: 8 *ToC*. The success encouraged us to evaluate this mutant for the treatment of simulated natural samples on a large scale. A mixture of five fatty acids, simulating the ratio of *cis*, *trans* and saturated fatty acids in partially hydrogenated vegetable oils (PHVO) (SI, Section 9), was tested on a 1 g scale. After irradiation for 1 h, the *trans* fatty acids were completely consumed, while only 9% conversion of *cis* fatty acids was observed, providing a *ToC* of up to 89:11 (Figure 5). Meanwhile 57% of saturated fatty acids were also converted. The important health benefits, satisfactory results, and potential applications for removing the *trans* fatty acids from edible oils highlight the importance of this strategy.

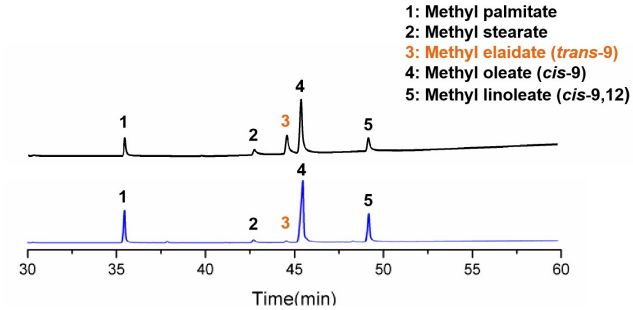

Figure 5. The transformation of PHVO mimic catalyzed by V453E. The GC Data was plotted by OriginPro 8. For the original chromatogram, please see the Supporting Information.

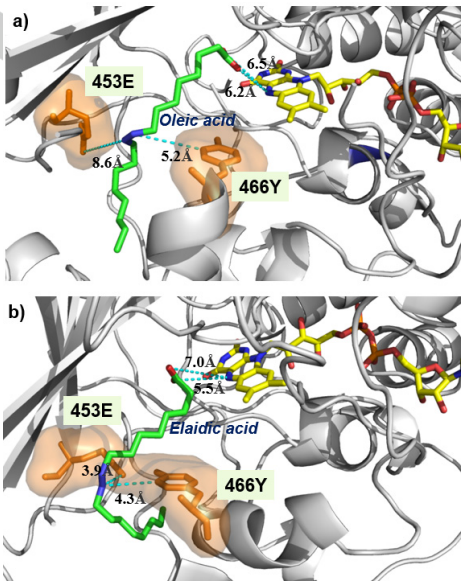

Figure 6. A typical snapshot of MD simulations for variant V453E with oleic acid (**a**) or elaidic acid (**b**) docked into the active site. The 453E and 466Y residue was orange, oleic acid and elaidic acid are colored green, with the key Δ9 double shown in blue.

To gain insight into the high *trans-cis* selectivity of the V453E mutant, we carried out molecular dynamics simulations for *Cv*FAP with oleic acid and elaidic acid. As the representative structures shown in Figure 6, the minimum distance between the oxygen atoms in elaidic acid and the N5 atom in FAD was shorter than that of oleic acid (5.5 Å for elaidic acid *vs.* 6.2 Å for oleic acid). Another significant difference was observed in the region for the position of the Δ9 double bond. Electron-rich residues, including

453E and 466Y, were stacking around the Δ9 double bond, leading to multiple p-π or π-π interactions that stabilized the binding of elaidic acid in the channel. Such interactions were greatly weakened when oleic acid was bound into the substrate channel due to the longer distance between the Δ9 double bond and both 453E and 466Y (Figure S6). In addition, we calculated the binding energy of oleic acid and elaidic acid with V453E, respectively. The result was consistent with the expected, displaying that elaidic acid was easily accepted by V453E with lower binding energy (-63.50 ± 0.53 kcal·mol^{-1} for elaidic acid and -37.09 ± 1.07 kcal·mol^{-1} for oleic acid, Table S5). Compared with V453E, oleic acid and elaidic acid docked into WT only showed relatively small difference between their binding energies or the minimum distance between the oxygen atoms of acid substrates and the N5 atom in FAD (Table S5, Figure S5). Next, we performed fluorescence kinetic measurements to further investigate the mechanism of the preferred selectivity *towards cis-trans* isomers. For WT, the fluorescence decay of ^{1}FAD* [8b,10c] was significantly accelerated in the presence of elaidic acid or oleic acid, indicating the electron transfer (ET) from the substrate to FAD* (Figure S7a). For the V453E mutant with elaidic acid, ^{1}FAD* fluorescence quenching by ET from elaidic acid seems to occur with the same kinetics as in WT with elaidic acid (i.e., in ~300 ps) (Figure S7b). However, for the V453E mutant with oleic acid, ^{1}FAD* fluorescence decays monophasically with a time constant of ~3 ns. The lack of rapid decay indicates there is no productive ET (Figure S7b). Moreover, according to the LC-MS result from the free radical trapping experiments, TEMPO product was observed for the elaidic acid. In contrast, no corresponding TEMPO product for oleic acid was detected (Figure S9). These results implied that in the best V453E mutant with oleic acid, the ET from oleic acid anion to the ^{1}FAD* and the subsequent formation of fatty acid radical failed, thus revealing the source of the significant difference in the activity of the best variant towards *cis-trans* isomers.

In summary, we have established a novel method for directly removing *trans*-fatty acids in a mixture of *cis*- and *trans*-fatty acids by highly-selective *Cv*FAP mutant-catalyzed decarboxylation. A protein engineering strategy was implemented by evolutionary FRISM based on p-π or π-π stacking interactions at the chosen mutation sites to achieve the best variant without a large screening workload. The best mutant, V453E, showed one-thousand-fold higher *trans-over-cis* selectivity for the model substrates than WT. Good results were also obtained in the gram-scale treatment of *trans*-fatty acids in a simulated partially-hydrogenated vegetable oil sample. MD simulations and spectroscopy experiments provided insight into the origin of the high *trans-over-cis* selectivity of the best mutant. This method provides a valuable protocol in the food safety field to prevent the excess consumption of *trans* fatty acids. The expansion of this method's scope to other types of edible oils is in progress in our laboratory.

Acknowledgements

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Keywords: Photo-decarboxylase • *trans* fatty acids • rational design • protein engineering • selectivity

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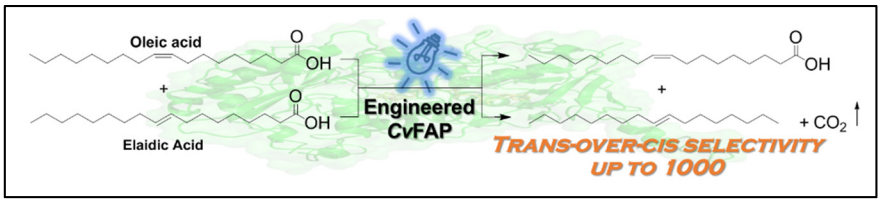

Highly-efficient removal of *trans* fatty acid from edible oils through decarboxylation catalyzed by engineered Fatty Acid Photo-decarboxylase (*Cv*FAP) is reported for the first time. The best mutant with a one-thousand-fold higher *trans-over-cis* selectivity for the model substrates than WT, was obtained through a rational engineering approach based on the FRISM strategy.