Enzyme Catalysis

A Photoclick-Based High-Throughput Screening for the Directed Evolution of Decarboxylase OleT

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Abstract: Enzymatic oxidative decarboxylation is an up-and-coming reaction yet lacking efficient screening methods for the directed evolution of decarboxylases. Here, we describe a simple photoclick assay for the detection of decarboxylation products and its application in a proof-of-principle directed evolution study on the decarboxylase OleT. The assay was compatible with two frequently used OleT operation modes (directly using hydrogen peroxide as the enzyme’s co-substrate or using a reductase partner) and the screening of saturation mutagenesis libraries identified two enzyme variants shifting the enzyme’s substrate preference from long chain fatty acids toward styrene derivatives. Overall, this photoclick assay holds promise to speed-up the directed evolution of OleT and other decarboxylases.

Incorporation of biobased resources into the value chain is of great importance in the context of a more sustainable society. In the recent past, synthetic routes have been explored to produce molecules of industrial relevance from renewable feedstocks. Carboxylic acids are ubiquitous in nature and their oxidative decarboxylation gives access to olefins, which are fundamental in chemical synthesis, e.g., as precursors in natural compound synthesis or as starting material for polymerization reactions. However, producing olefins from natural carboxylic acids is difficult by chemical means. Most of the reactions involve radical formation (e.g., Hunsdiecker reaction[1] or Kolbe electrolysis)[2] and therefore produce side products. Another disadvantage is the use of toxic or expensive metalorganic species for decarboxylation reactions (e.g., Kochi reaction[3] or Barton decarboxylation).[4] Thus, mild and catalytic oxidative decarboxylations are sought-after reactions.

OleT from Jeotgalicoccus sp. ATCC 8456 is a P450 peroxynase of the CYP152 family.[5] It catalyzes the oxidative decarboxylation of long chain fatty acids using hydrogen peroxide as oxidant,[6] which can either be added directly to the reaction or produced in situ.[7] Alternatively, OleT was successfully combined with surrogate redox partners[7c, 8] or genetically fused to reductase domains.[8b]

Previously, protein engineering was used to widen the substrate scope of OleT toward aromatic carboxylic acids. However, due to low protein expression levels and the lack of a high-throughput assay, OleT variants had to be produced in a bigger scale and screened using chromatographic methods.[8c] In search of a simple plate reader assay for the detection of small terminal alkenes, a study by Song et al. drew our interest. They fluorescently labeled a non-canonical amino acid containing a terminal olefin moiety (O-allyl-tyrosine)[9] using diarylterazoles by forming a fluorescent cycloadduct[10] (Scheme 1A).

Inspired by that work, we set out to develop a photoclick-chemistry-based high-throughput screening assay to interrogate mutagenesis libraries of the decarboxylase OleT for

Scheme 1. Diarylterazole 1 as photoclick reagent.
Among the residues in the vicinity of OleT-BM3R, we chose OleT-BM3R. OleT-BM3R was recently described by Lu et al. as a fusion protein of the P450 decarboxylase OleT and the Bacillus megaterium P450-BM3 reductase domain (termed BM3R; P450-BM3 residues 451–1048; Figure 1A). We considered OleT-BM3R to be the ideal candidate as it can be run in two different operational modes, both of which are frequently used in OleT studies: (i) the hydrogen peroxide-driven mode ("peroxide mode"), and ii) the reductase-driven mode ("reductase mode"); Figure 1B). In the peroxide mode, hydrogen peroxide is added and initiates Compound I formation in the OleT active site. In the reductase mode, a reducing equivalent (here: NADPH) is used and a reductase (here: BM3R) shuttles electrons from the reducing equivalent toward OleT leading to its activation.

We prepared two identical 96-well plates. One half of the wells of each plate were inoculated with E. coli cells harboring Ole-BM3R; the other half contained an empty vector control. After cell lysis and centrifugation, the cleared lysates were applied for the decarboxylation of 3-(4-bromophenyl)propanoic acid (5a) using the peroxide mode (one plate) and the reductase mode (the other plate), respectively. In the latter case, the reaction was combined with a cofactor recycling system using an engineered variant of phosphite dehydrogenase (PTDH; Figure 1B). After overnight incubation, the plates were subjected to the photoclick assay (detailed procedures in Supporting Information). In both reaction modes the wells containing Ole-T-BM3R exhibited higher fluorescence than the empty vector control with a coefficient of variance (CV) of 15% and 14% for the peroxide mode and the reductase mode, respectively, and a strictly standardized mean difference (SSMD) of 4.2 and 5.3, respectively (Figure S2, Supporting Information). A CV ≤ 15% is frequently and successfully used in directed evolution experiments and a cutoff criterion of SSMD > 3 is often used to evaluate high-throughput assay quality.

Westem blots were performed with results shown in Figure 2. OleT-BM3R was efficiently expressed in both the peroxide and reductase modes.

With conditions for a microplate photoclick assay in hand, we set out to perform a proof-of-principle directed evolution study on OleT-BM3R to improve the enzyme’s selectivity for substrate 5a. We started by computationally docking 5a into the active site of the OleT crystal structure (details in Supporting Information) to identify key amino acids involved in the binding of 5a. The molecular docking study revealed that the substrate binds close to the heme cofactor with a binding energy of −7.66 kcal mol⁻¹ and forms two hydrogen bonds with R245 (Figure S3, Supporting Information), much like the hydrogen bonds formed with the natural fatty acid substrates. Among the residues in the vicinity of 5a (Figure S3, Supporting Information), residue L78 of OleT-BM3R was subjected to saturation mutagenesis. The mutant library was screened for the conversion of 5a using the established photoclick assay. Unfortunately, most of the generated variants were inactive as determined by the photoclick assay and the...
active variants did not show an improvement over wildtype (Figure S4, Supporting Information). We proceeded by saturating F79 of OleT-BM3R. According to our docking model, F79 is in close contact with 5a (5.6 Å between aromatic rings of F79 and 5a) and previous studies suggested the importance of this position for substrate selectivity. Two potential “hits” revealed the F79L and F79V substitutions, respectively, and both variants were found to have improved decarboxylation activity of 5a in a confirmatory validation experiment at larger scale (shaking flask expression) using cleared lysates (Figure 2).

The F79L substitution improved the total turnover number 1.8-fold (219 TTN) with respect to the OleT-BM3R parent (123 TTN; Figure 2). This substitution was previously reported by Wang et al. to improve the conversion of aromatic substrates. In their study, OleT was combined with the CamAB reductase system and the authors identified the F79L substitution after gas chromatography-based screening of a library with reduced amino acid alphabet. Indeed, our photoclick assay was able to discover a previously not reported variant, outperforming the active site, allowing hydrogen abstraction at the alternative way that favors hydrogen abstraction from the C-β of OleT-BM3R, to the in situ formation of hydrogen peroxide rather than the direct reduction of dioxygen. Accordingly, Compound I formation and turnover are attributed to hydrogen peroxide rather than the direct reduction of dioxygen at the heme. Our study corroborates these findings, suggesting that the in situ formation of hydrogen peroxide plays an important role also in the activation of OleT-BM3R in “reductase mode” and that, unlike in P450-BM3, the electron transfer between reductase domain and OleT is not optimally tuned. In order to maximize total turnovers, however, omitting catalase and thus deliberately allowing in situ hydrogen peroxide formation renders OleT-BM3R a much more productive decarboxylation system. To study the substrate scope of OleT-BM3R and the F79L and F79V variants, reactions were carried out in the absence of catalase (Table 1).

In general, the purified enzymes performed better compared to the set-up in cleared lysate (compare Table 1, entry 1 and Figure 2), which we attribute to the presence of host cell catalases in the lysates. Notably, the purified F79V variant exhibited 1.3-fold improved activity compared to the OleT-BM3R parent using substrate 5a (3.7-fold for the reaction set-up in cleared lysates), whereas the F79L variant was less active than OleT-BM3R (vs. 1.8-fold improvement in lysates). Differences in performance in whole cells/cell lysates vs. purified enzymes are not uncommon, which is why validation of promising hits in vitro is often necessary. They are often governed by the stabilizing effects present in cells and lysates. Among the 3-phenylpropanoic acid derivatives (2a–6a, entry 1–5) the different OleT-BM3R variants exhibited different preferences: Unsurprisingly, F79V preferred the bromo-substituted substrate which was used in the microplate screening. F79L was the most efficient producer of the fluoro-substituted styrene derivative 3b. Overall, OleT-BM3R and the two variants have a broad substrate scope ranging from its native long chain fatty acid substrate (7a, entry 6) to shorter chain lengths (8a, entry 7) or even small cyclic compounds (9a, entry 8). Notably, the F79V variant lost its preference for fatty acid substrates (7a; 616 TTN) in favor of substrate 5a (1452 TTN). For the decarboxylation of the tetrahydronaphthalene derivative 10a we observed that the 10b:10c selectivity was tunable upon substituting F79 by either L or V (Table 1, entry 9 and footnote b). Looking at the OleT active site, it is conceivable that π-stacking interactions between F79 and substrate 10a orient the substrate in a way that favors hydrogen abstraction from the C-β that leads to the formation of 10b. Replacing F79 with the non-aromatic L or V could facilitate the movement of substrate 10a in the active site, allowing hydrogen abstraction at the alternative C-β and leading to increased formation of 10c.
Table 1. Comparative substrate scope of OleT-BM3R and its variants (F79L and F79V).

| Entry | Substrate | TTN | OleT-BM3R | F79L | F79V |
|-------|-----------|-----|-----------|------|------|
| 1     | 5a (R = Br) | 1084 | 886 | 1452 |
| 2     | 2a (R = H)  | 1200 | 758 | 530  |
| 3     | 3a (R = F)  | 750  | 965 | 635  |
| 4b    | 4a (R = Cl) | 765  | 704 | 849  |
| 5     | 6a (R = OMe) | 269  | 239 | 243  |
| 6b    |             | 2290 | 1402 | 616  |
| 7     |             | 151  | 94  | 59   |
| 8     |             | 116  | 56  | 14   |
| 9     |             | 690b[c] | 486bc | 412bc | |
| 10    |             | 52b[c] | 37b[c] | 29b[c] | |
| 11    |             | 0    | 0    | 0    | |
| 12    |             | 0    | 0    | 0    | |

Reaction conditions (reductase mode): 1 mM carboxylic acid substrate, 0.5 μM purified OleT-BM3R (or variants thereof), 200 μM NADPH, 10 mM sodium phosphate, 5 mM PTDH, 5% DMSO as cosolvent, 16 h, 23 °C. No oxo-transfer side products (e.g., hydroxylation of substrate or epoxidation of styrenyl product) were observed. [a] 0.05 μM purified OleT-BM3R (or variants thereof) were used. [b] 1,2-dihydronaphthalene (10b) and 1,4-dihydronaphthalene (10c) were formed, respectively. 10b:10c ratios were: OleT-BM3R = 99:1; F79L = 93:7; F79V = 87:13. [c] Kinetic resolution of the substrate was not observed. (d) 2-pentene (11b) was formed as trans- and cis-isomer. trans:cis ratios were: OleT-BM3R = 98:2; F79L = 98:2; F79V = 97:3. 1-pentene was not observed. [e] No single- or double-decarboxylation products were detected.

minor changes in the trans:cis selectivity were observed (Table 1, entry 10 and footnote d). Moreover, 1-pentene was not detected. The branched chain substrate 12a and the dicarboxylic acid 13a were not converted by any of the three enzyme variants (entries 11 and 12) although double-decarboxylation has been previously reported for other substrates.[11]

In summary, we developed a robust 96-well photoclick assay for the quantitative detection of styrene derivatives in the micromolar range and potentially other alkene-products resulting from decarboxylation reactions (e.g., derivatives of the O-allyltroisine initially used by Song et al.)[10] The simple handling and convenient fluorescent read-out enable high-throughput screening of comprehensive decarboxylase variant libraries within hours. Moreover, the assay can be performed with minimal instrumentation, as most laboratories active in the field of directed evolution are already equipped with UV transilluminators. In a proof-of-principle directed evolution study, we applied our assay to change the substrate specificity of the long chain fatty acid preferring OleT-BM3R fusion enzyme toward the conversion of a non-natural aromatic substrate. This study has potential implications for the directed evolution of OleT and other decarboxylases,[20,24] in particular, for changing the enzyme’s substrate selectivity, its resistance toward hydrogen peroxide, or for fine-tuning the hitherto suboptimal electron transfer of reductase partner systems to OleT.

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Conflict of interest

The authors declare no conflict of interest.

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