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The power of phylogeny: Rational redesign of bacterial eugenol oxidases towards non-natural substrates

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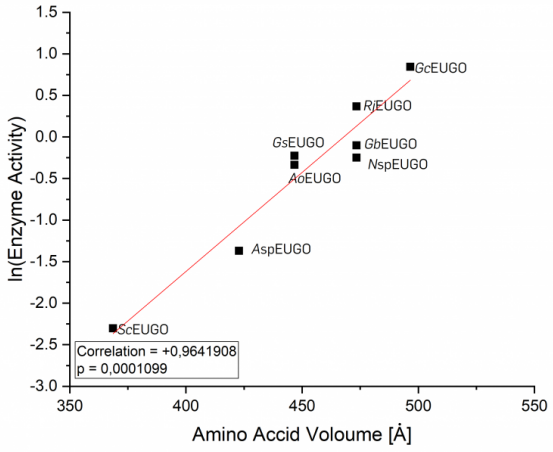
PURPOSE OF THE ABSTRACT
Bacterial eugenol oxidases (EUGOs) and their fungal homologues, vanillyl alcohol oxidases (VAOs), form an enzyme family of versatile biocatalysts.[1,2] They catalyze a variety of different reaction types on a broad substrate range.[3] The oxidation of alkanes or alcohols in benzylic position is the characteristic reaction of both subfamilies including the oxidation of eugenol and vanillyl alcohol.[4,5] As phenolic compounds are important building blocks in the chemical industry for polymers, fragrances or flavors, we intended to widen the substrate scope towards none-natural substrates. Therefore, we created an R-based GUI-driven software to streamline enzyme mining and identify crucial residues in the enzyme structure. These positions were explored by mutagenesis, and the crystal structure of the most promising candi-date was obtained.

The use of computer-guided approaches in the field of enzyme design increases in popularity and recently great success was achieved by tailoring the eugenol oxidase from Rhodococcus jostii RHA1 (RjEUGO) through an in silico strategy to produce iso-eugenol.[6] Despite this recent advancement, the overall sequences space of EUGOs is rather poorly investigated with the mentioned RjEUGO representing the only member so far. Thus, we explored homologous enzymes in order to find naturally improved variants.

Among the enzyme family, we compared key residues and selected a set of seven candidates for subsequent characterization. The substrate profile of each enzyme was correlated with a respective structural model. As example, the sterically size of three residues was found to effect the conversion of 3-bromo-4-hydroxybenzyl alcohol (Figure 1), a motive commonly found in bromo dyes like pH indicators. Subsequently, we performed site-saturation mutagenesis screening ~100 clones for every position individually (>95% library coverage) using a hydrogen peroxide sensitive assay in the cell-free crude extract. Characterization of the best enzyme variant highlighted a nine-fold improved vmax (14.7 ± 0.5 U/mg) compared to the wild type and even higher activities compared to other EUGOs (Figure 2).

In our effort to explore non-natural substrates for EUGOs, we obtained sixteen active enzyme variants and achieved improved reaction rates compared to the respective most active natural enzyme for the dehydrogenation of 4-cyclopentylphenol (2.5-fold, ScEUGO V427I L282M), the deamination of vanillyl amine (2.2-fold, ScEUGO E378T), the ether cleavage of butyl vanillyl ether (2.5-fold, ScEUGO V427I) and, as a side effect, for the hydroxylation of eugenol (1.4-fold, ScEUGO V427I). As the enzyme from Streptomyces cavernae (ScEUGO) appeared as the overall most interesting enzyme, we obtained the crystal structure up to 1.8 Å resolution to validate our models and explain the observed effects on a molecular level. Thus, we managed to construct robust relationships between structure and function of EUGOs. Crucial residues for selectivity were identified and can be used to design a tailored oxidase. Further, the presented computational tools allow a transfer of this methodology to other enzyme families and will significantly improve the quality and pace of enzyme mining.

FIGURES



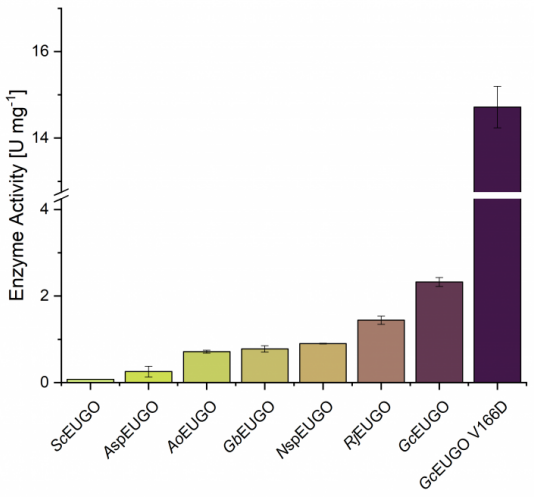


FIGURE 1

Activity correlation

Semi-logarithmic plot of the enzyme activity on 3-bromo-4-hydroxybenzyl alcohol of indicated EUGOs against the added volume of amino acids in position 166, 391 and 427 (RjEUGO numbering). Enzyme activity increases with the sterical demand of these residue

FIGURE 2

Mutagenesis

Enzyme activity on 3-bromo-4-hydroxybenzyl alcohol by natural enzymes in comparison with GcEUGO V166D. The variant was found to be most active in the mutagenesis studies.

KEYWORDS

enzyme mining | enzyme engeering | structure-function relations | side-saturation mutagenesis

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