A 96-multiplex capillary electrophoresis screening platform for α-isophorone oxidation by P450 BM3

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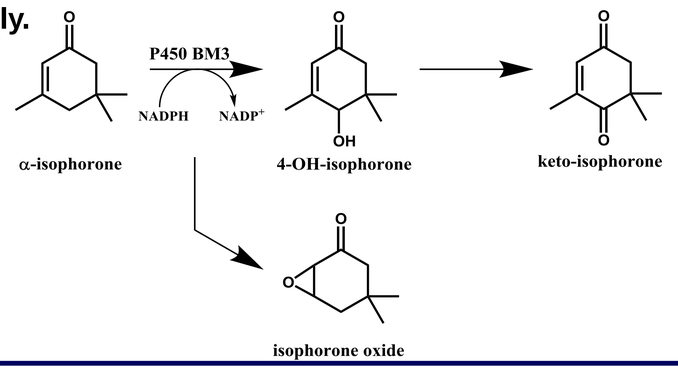
Introduction

Mutation library screening of NADPH dependent enzymes is frequently performed by a cofactor depletion assay determining initial activities [1]. However, determined activities do not only account for target product formation alone, they also include contributions of side product formation. In case of P450 monooxygenases use of unnatural substrates often increases the so called uncoupling which describes inefficient electron usage leading to reactive oxygen species (ROS: H_2O_2, ·OH) instead of target product formation and aggravates the selection of truly improved variants.

One promising analytical tool to quantify product formation and avoid false selections is capillary electrophoresis (CE) with its universal product detection possibilities (UV-detector).

Separation is achieved by differential migration of solutes in an electric field. To accomplish separation of neutral species the use of surfactants in the running buffer was introduced by Terabe in 1984 [2], named micellar electrokinetic capillary electrophoresis (MEKC). Multiplex systems even allow the simultaneous measurement in 96-well format.

A MEKC screening platform was established and compared to the conventional NADPH depletion screening within evolution efforts towards improved P450 BM3 variants which form 4-hydroxy-isophorone from *α*-isophorone in higher amounts and more selectively.



Experimental

CE screening system validation

Multiplexed capillary electrophoresis allows the analysis of 96 samples in one run. Combined with the possibility of detection and quantification of several compounds, this system makes a promising HTS method for directed evolution approaches.

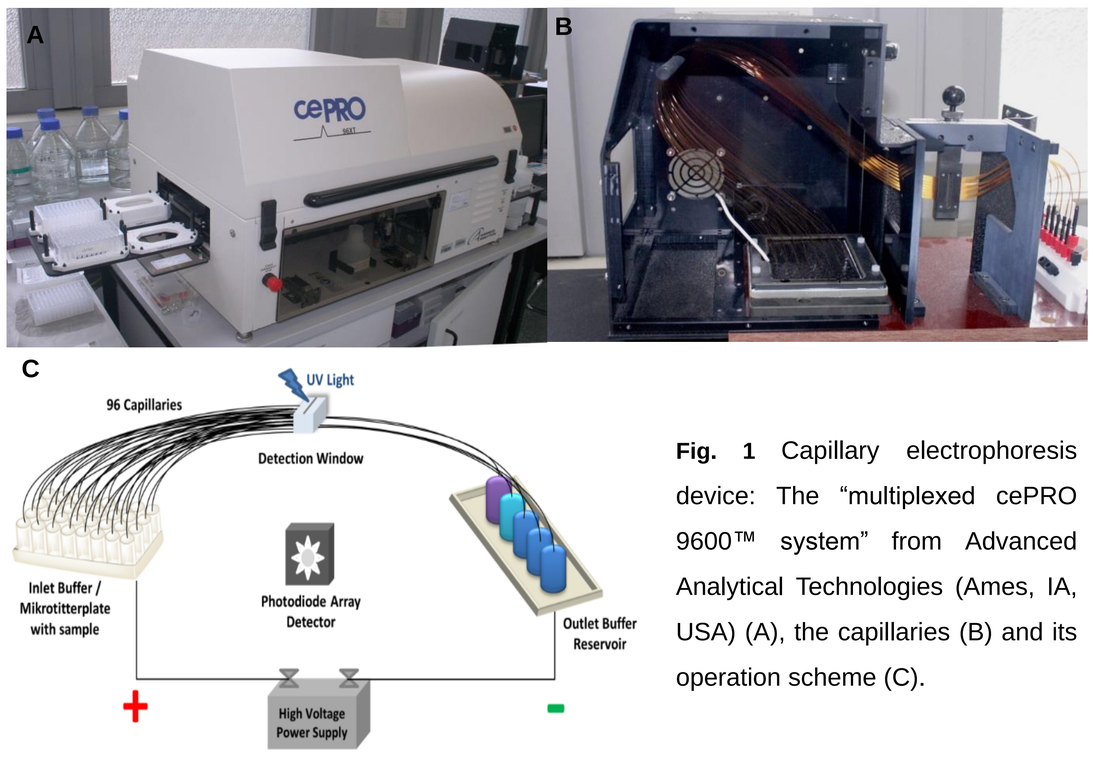


Fig. 1 Capillary electrophoresis device: The "multiplexed cePRO 9600™ system" from Advanced Analytical Technologies (Ames, IA, USA) (A), the capillaries (B) and its operation scheme (C).

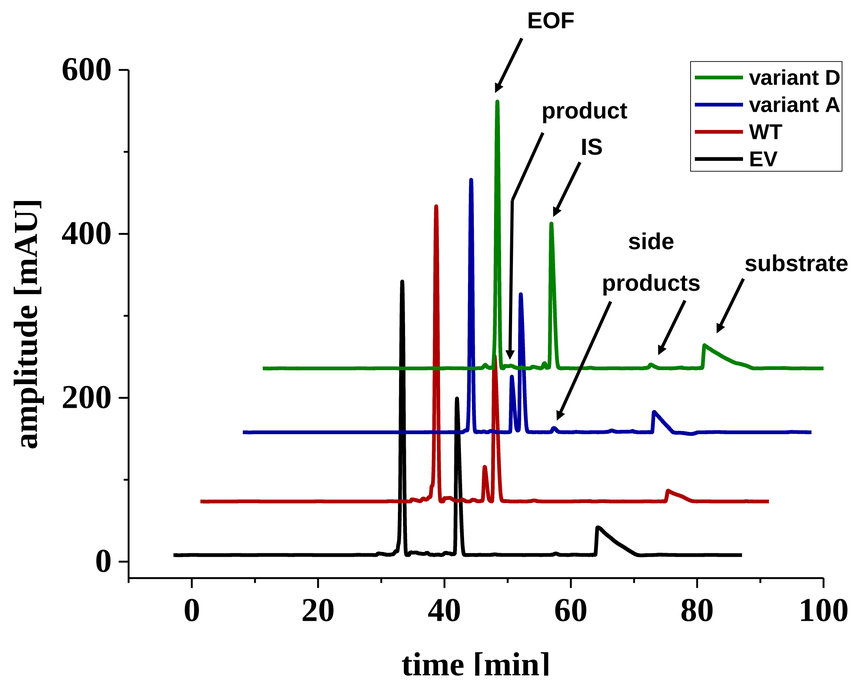


Fig. 2
Capillary electrophoresis electropherograms of different variants showing their product profil. [3]
WT: P450 BM3 wild type
EV: empty vector
EOF: electroosmotic flow, **IS**: internal standard, **substrate**: α-isophorone, **product**: 4-OH-isophorone, **side products**: keto-isophorone and isophorone oxide

- Differences in product formation are well recognized within the system [Fig. 2] and can even be quantified which allows variant selection based on specific target product formation.
- The system shows a low standard deviation of 12.1% (product formation of 4-hydroxy-isophorone by P450 BM3 wild type), which enables reliable screening of evolution libraries.

Variant characterization

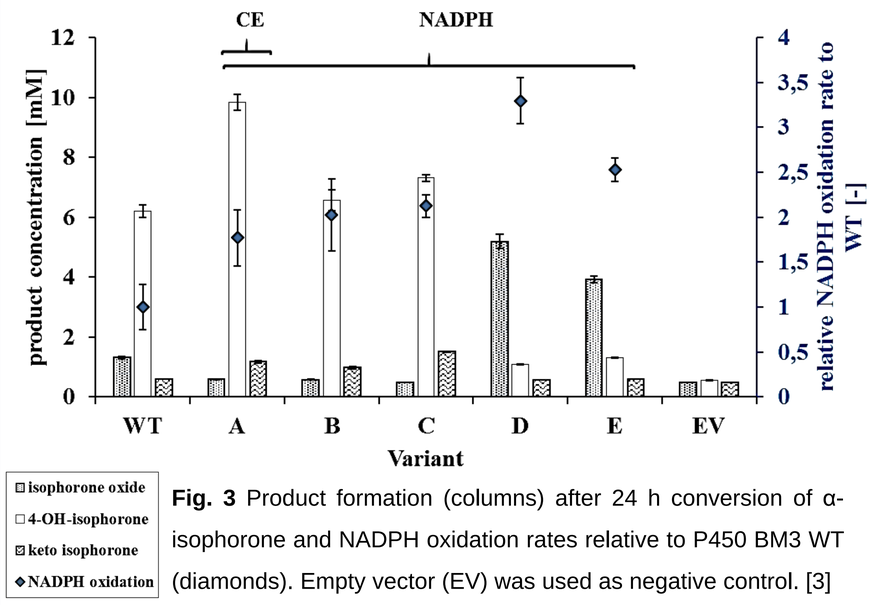


Fig. 3 Product formation (columns) after 24 h conversion of α-isophorone and NADPH oxidation rates relative to P450 BM3 WT (diamonds). Empty vector (EV) was used as negative control. [3]

Two libraries were screened with the NADPH depletion based and the new CE based system. All variants with varying substitutions found within both screening systems were compared in terms of their product formation. **[Fig. 3]**

- **CE based screening** led to one variant (**A**) with increased NADPH oxidation and 4-OH-isophorone formation.
- **NADPH based screening** led to 5 variants (**A-E**) with increased NADPH oxidation and of which only 3 (A-C) showed increased 4-OH-isophorone formation.

Conclusion

Multiplexed Capillary Electrophoresis

- allows the exclusion of variants with undesired products and identification of variants with true formation of desired products ahead of larger scale characterization studies. **[Fig. 2, 3]**
- reduces the time related effort in finding improved and robust biocatalysts.
- is a novel and highly valuable screening platform to identify more efficient P450 BM3 variants.

References

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[2] Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. (1984) Electrokinetic Separations with Micellar Solutions and Open-Tubular Capillaries. *Anal. Chem*. 1984, 56, 111-113.

[3] Gärtner, A.; Ruff, A. J.; Schwaneberg, U. (2018) A 96-Multiplex Capillary Electrophoresis Screening Platform for α-Isophorone Oxidation by P450 BM3. *drafted*.

Acknowledgements

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