DE GRUYTER Z. Naturforsch. 2019; 74(3–4)c: 101–104

Letter

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Multienzymatic in situ hydrogen peroxide generation cascade for peroxygenase-catalysed oxyfunctionalisation reactions

https://doi.org/10.1515/znc-2018-0137
Received September 4, 2018; revised September 19, 2018; accepted October 4, 2018

Abstract: There is an increasing interest in the application of peroxygenases in biocatalysis, because of their ability to catalyse the oxyfunctionalisation reaction in a stereoselective fashion and with high catalytic efficiencies, while using hydrogen peroxide or organic peroxides as oxidant. However, enzymes belonging to this class exhibit a very low stability in the presence of peroxides. With the aim of bypassing this fast and irreversible inactivation, we study the use of a gradual supply of hydrogen peroxide to maintain its concentration at stoichiometric levels. In this contribution, we report a multienzymatic cascade for in situ generation of hydrogen peroxide. In the first step, in the presence of NAD^+ cofactor, formate dehydrogenase from *Candida boidinii* (FDH) catalysed the oxidation of formate yielding CO_2. Reduced NADH was reoxidised by the reduction of the flavin mononucleotide cofactor bound to an old yellow enzyme homologue from *Bacillus subtilis* (YqjM), which subsequently reacts with molecular oxygen yielding hydrogen peroxide. Finally, this system was coupled to the hydroxylation of ethylbenzene reaction catalysed by an evolved peroxygenase from *Agrocybe aegerita* (r*Aae*UPO). Additionally, we studied the influence of different reaction parameters on the performance of the cascade with the aim of improving the turnover of the hydroxylation reaction.

Keywords: formate dehydrogenase; hydrogen peroxide generation; old yellow enzyme; oxyfunctionalisation; peroxygenase.

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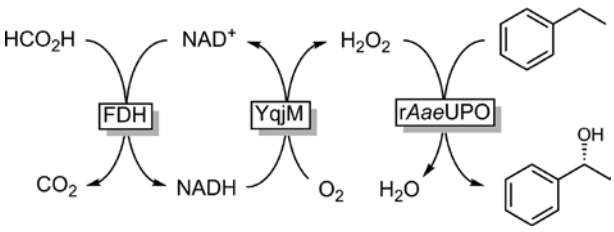
Fungal peroxygenases (E.C. 1.11.2.4) are promising catalysts for the selective oxyfunctionalisation of non-activated C–H- and C=C-bonds [1–5]. Compared with the well-known P450 monooxygenases, peroxygenases excel by their simpler reaction mechanism and minor requirements, as they are secreted – extracellular – enzymes. Instead of reductively activating molecular oxygen at the expense of NAD(P)H and sometimes rather complex and vulnerable electron transport chains [6], peroxygenases utilise H_2O_2 directly through a H_2O_2-shunt pathway [7]. This approach, however, is challenged by the poor robustness of heme-dependent enzymes against the strong oxidant H_2O_2. To alleviate this, catalytic reduction of O_2 is the most commonly used approach nowadays. As a source of reducing equivalents, the cathode [8–10] glucose and other small molecules have been reported [11–17].

Inspired by a contribution by Guisan et al. [18] we decided to evaluate formic acid (or its sodium salts) as stoichiometric reductant to promote peroxygenase-catalysed oxyfunctionalisation reactions. The system envisages the coupling of formate dehydrogenase (FDH)-catalysed [19, 20] oxidation of formic acid to the reduction of molecular oxygen to hydrogen peroxide, utilising the NAD(P)H oxidase activity of old yellow enzymes such as the one from *Bacillus subtilis* (YqjM) [21, 22]. In previous studies, it was demonstrated that YqjM, in the absence of a reducible conjugated C=C double bond, functions also as a NADH oxidase. Hence, the combination of FDH (formate consuming and NADH regenerating) with YqjM (NADH consuming and H_2O_2 generating) resulted in an artificial, bienzymatic formate oxidase system. Both reactions are being coupled via the natural redox cofactor (NAD^+/NADH). As the model reaction to establish the system, the stereoselective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol (Scheme 1) catalysed by the recombinant peroxygenase from *Agrocybe aegerita* (r*Aae*UPO, -PaDa-I mutant-), a variant evolved in the laboratory for functional expression in yeasts [23, 24], was used.



Scheme 1: Proposed in situ H_2O_2 generation system to promote the stereoselective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol using the recombinant peroxygenase from *A. aegerita* (r*Aae*UPO). In situ H_2O_2 generation is achieved by reductive activation of O_2 through a cascade of FDH-catalysed oxidation of formic acid and concomitant reduction of nicotinamide adenine dinucleotide (NAD^+) to its reduced form (NADH). The latter is reoxidised by the old yellow enzyme homologue from *B. subtilis* (YqjM) to reduce O_2 to H_2O_2.

The relevant enzymes for this study (i.e., FDH, YqjM and r*Aae*UPO) were recombinantly expressed in *Escherichia coli* (FDH and YqjM) and *Pichia pastoris* (r*Aae*UPO). Details for the expression conditions, yields and purification protocols can be found in the Supporting Information. In essence, it was most important to remove any catalase activity from the *E. coli*-derived enzyme preparations as this would interfere with the r*Aae*UPO-catalysed oxyfunctionalisation reaction.

Having the catalase-free enzyme preparations at hand, we further characterised the crucial parameters for FDH for formate-driven NADH regeneration and YqjM-catalysed aerobic reoxidation of NADH, yielding H_2O_2 as the desired product. In accordance with the literature [25], FDH exhibited a medium affinity towards formate ($K_M = 13.5 \pm 1.1$ mM, Figure S3 and Table S2). Therefore, to avoid rate limitations caused by low formate concentrations, we used at least 75 mM initial concentrations of formate. Both, YqjM and FDH showed sufficient activity at slightly alkaline pH values, which had previously been shown to be optimal for r*Aae*UPO for benzylic hydroxylation reactions (Table 1) [26].

To test our system, we combined the three enzymes and cofactor in one pot in the presence of ethyl benzene (Figure 1). It is worth mentioning that in all experiments

Table 1: Activity of YqjM and FDH at different pH values.

pH	A_{spec} (YqjM), U/mg[a]	A_{spec} (FDH), U/mg[b]
7	0.51 ± 0.02	1.58 ± 0.02
7.5	0.64 ± 0.01	1.98 ± 0.06
8	0.65 ± 0.01	2.21 ± 0.04

[a]General conditions: 0.5 mM NAD^+; 0.1 mg/mL FDH; 150 mM sodium formate; 50 mM KPi; 30 °C. [b]General conditions: 0.15 mM NADH; 50 mM KPi; 30 °C.

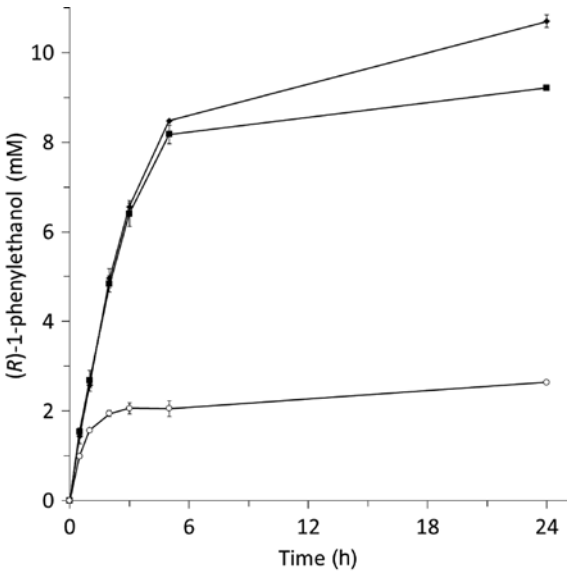


Figure 1: Influence of the volume ratio of air to reaction mixture: ♦, 3:1; ■, 1:1; and ○, 1:4. Conditions: 3-(N-Morpholino) propanesulfonic acid (MOPS)-buffer (50 mM, pH 7), c(ethylbenzene) = 50 mM, c(methanol) = 200 mM, c(NAD^+) = 0.5 mM, c($NaHCO_3$) = 75 mM, c(r*Aae*UPO) = 50 nM, c(FDH) = 2.5 μM, c(YqjM) = 2.67 μM; *T* = 30 °C; 600 rpm. For reasons of clarity, the 'overoxidation product' acetophenone as well as the remaining ethyl benzene were omitted.

reported here, the enantiomeric excess (ee) of the product ((*R*)-1-phenyl ethanol) was higher than 96% ee, thereby representing the normal enantioselectivity of r*Aae*UPO for this reaction. It is worth noting here that because of the high volatility of the starting material (ethyl benzene), comparably high initial concentrations were applied to compensate for the evaporation loss of the reagents. To attain such elevated concentrations, we used 0.8% (v/v) of methanol as a cosolvent. Control reactions in the absence of either of the catalysts (FDH, NAD, YqjM, or r*Aae*UPO) yielded no detectable product formation; the same is true for experiments performed in the absence of formate or under anaerobic conditions (Table S1).

Under arbitrarily chosen conditions, enantioselective conversion of ethyl benzene into (*R*)-1-phenyl ethanol was observed. However, only 2.5 mM of the desired product was obtained, suggesting a limitation in O_2 availability. Therefore, the headspace volume was increased (Figure 1, ♦ and ■). Using a phase ratio of 1:1 (ambient air to reaction mixture), approx. 9.6 mM of (*R*)-1-phenyl ethanol may be expected (calculated from the O_2 availability in the gas phase plus O_2 dissolved in buffer), which is also roughly the product concentration observed under these conditions (Figure 1, ■). However, further increases of the headspace volume did not result in the expected increases in product formation.

We suspected other factors, particularly enzyme stability, to account for the lower than expected performance. Therefore, we investigated the influence of the catalytic components of Scheme 1 more systematically (Table 2). In general, increasing the concentration of either catalyst (FDH, NAD, or YqjM) increased the initial rate of the overall reaction and consequently influenced the turnover number of the other catalysts. Compared with this, the initial rate of the overall reaction was rather insensitive towards changing r*Aae*UPO concentrations. Depending on the reaction, turnover numbers of 80,000–390,000 for r*Aae*UPO, 14,000–84,000 for FDH, 1600–6700 for YqjM and 7–69 for NAD were observed.

This observation was rather expected considering the very high catalytic activity of r*Aae*UPO under initial rate conditions of 410 s^{-1} [26]. Hence, the overall reaction appeared to be limited by the availability of H_2O_2. Its concentration, however, cannot be increased at will as too much H_2O_2 will also irreversibly inactivate r*Aae*UPO. Therefore, we followed the operational stability of the overall process in the presence of different FDH concentrations.

As shown in Figure 2, the product accumulation ceased earlier in the presence of higher FDH concentrations. Hence, FDH activity plays a crucial role for both the productivity and the robustness of the overall system. Future studies will have to be undertaken to optimise the relative concentrations of the catalysts used. A design of an experimental approach appears promising to find the optimal conditions of this highly interconnected reaction system. Also, it should be very interesting to identify the amino acid residues prone to oxidative modification and substitute them by less reactive ones, eventually yielding more robust FDH mutants.

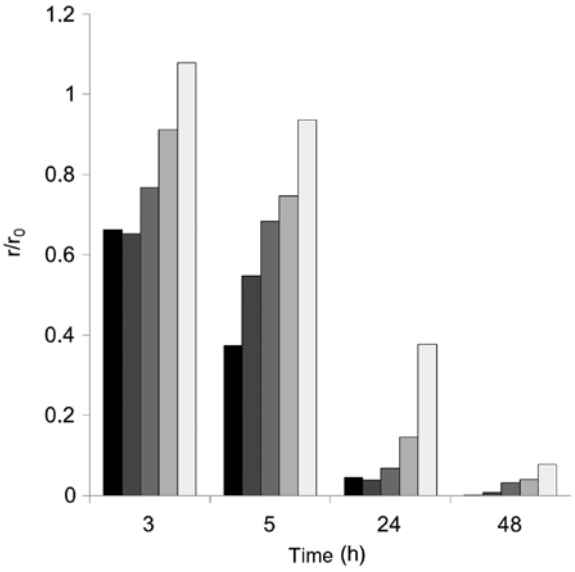


Figure 2: Effect of FDH concentration on the robustness of the overall reaction. Reaction rates at different time intervals were normalised by corresponding initial reaction rates. Conditions: MOPS-buffer (50 mM, pH 7), c(ethylbenzene) = 50 mM, c(methanol) = 200 mM, c(NAD^+) = 0.5 mM, c($NaHCO_2$) = 75 mM, c(r*Aae*UPO) = 50 nM, c(YqjM) = 2.67 μM; *T* = 30 °C; 600 rpm. c(FDH) = 0.25 μM (■), 0.63 μM (■), 1.25 μM (■), 1.87 μM (□) and 2.5 μM (□). The initial rates were 1.1, 1.8, 2.6, 3.0 and 2.6 mM/h, respectively.

Overall, in this contribution, we have provided the proof-of-concept for a FDH/NAD/YqjM cascade to mediate the reductive activation of molecular oxygen to hydrogen peroxide. Promising turnover numbers for all catalytic

Table 2: Influence of the different reaction parameters on initial reaction rates and turnover numbers.

	Concentration, mM	v_0, mM/h	Turnover number (mol product/mol catalyst)			
			FDH	NAD^+	YqjM	r*Aae*UPO
[FDH], nM[a]	50	0.36	84,000	8.4	1600	80,000
	125	0.66	47,000	11.9	2200	120,000
	250	0.96	26,000	13.1	2500	130,000
[NAD^+], mM[b]	0.1	0.52	28,000	68.8	2600	140,000
	0.25	0.76	30,000	30.3	2800	150,000
	0.5	0.96	26,000	13.1	2500	130,000
[YqjM], μM[c]	0.53	0.15	14,000	7.1	6700	70,000
	2.67	0.96	26,000	13.1	2500	130,000
	13.33	1.78	38,000	19.2	700	190,000
[r*Aae*UPO], nM[d]	25	0.83	39,000	19.3	3600	390,000
	50	0.96	26,000	13.1	2500	130,000
	100	0.78	34,000	17	3200	90,000

Conditions: MOPS-buffer (50 mM, pH 7), [ethylbenzene] = 10 mM, [methanol] = 200 mM, [$NaHCO_2$] = 75 mM; *T* = 30 °C; 600 rpm. [a][NAD^+] = 0.5 mM, [r*Aae*UPO] = 50 nM, [YqjM] = 2.67 μM. [b][FDH] = 250 nM, [r*Aae*UPO] = 50 nM, [YqjM] = 2.67 μM. [c][NAD^+] = 0.5 mM, [r*Aae*UPO] = 50 nM, [FDH] = 250 nM. [d][NAD^+] = 0.5 mM, [FDH] = 250 nM, [YqjM] = 2.67 μM.

components have been achieved, but significant improvements are necessary to turn this system truly practical. For this, the identification of the limiting factors as provided in this study will lay the foundation.

Acknowledgements: Financial support by the European Research Council (ERC Consolidator Grant No. 648026) is gratefully acknowledged.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/znc-2018-0137).