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Convergent Cascade Catalyzed by Monooxygenase–Alcohol Dehydrogenase Fusion Applied in Organic Media

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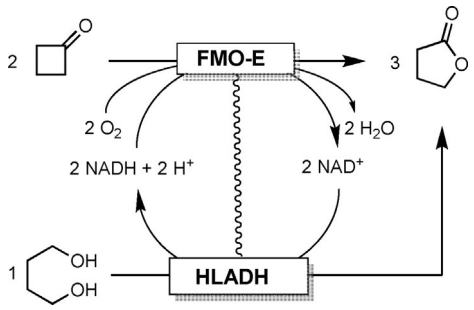
With the aim of applying redox-neutral cascade reactions in organic media, fusions of a type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) were designed. The enzyme orientation and expression vector were found to influence the overall fusion enzyme activity. The resulting bifunctional enzyme retained the catalytic properties of both individual enzymes. The lyophilized cell-free extract containing the bifunctional enzyme was applied for the convergent cascade reaction consisting of cyclobutanone and butane-1,4-diol in different microaqueous media with only 5% (*v*/*v*) aqueous buffer without any addition of external cofactor. Methyl *tert*-butyl ether and cyclopentyl methyl ether were found to be the best organic media for the synthesis of γ-butyrolactone, resulting in about 27% analytical yield.

Nature uses elegant synthetic strategies by coupling enzymes in metabolic pathways, in which the product of one enzyme is the substrate of the next enzymatic reaction. The catalytically related enzymes often form complexes to increase the efficiency of these enzymatic cascade reactions, such as the pyruvate dehydrogenase complex.[1] The design of artificial multienzymatic reactions has been of great interest in biocatalysis during recent decades.[2–4] Cascade reactions have become attractive, especially for redox biocatalysis, because internal cofactor regeneration can be achieved; thus creating self-sufficient redox reactions.[5–7] A nicotinamide adenine dinucleotide (NADH)-dependent redox-neutral convergent cascade reaction composed of a recently discovered type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) has been established in our previous work.[8] Two model cascade reactions were analyzed for the synthesis of γ-butyrolactone and chiral bicyclic lactones. In the targeted cascade reaction, FMO-E catalyzes the Baeyer–Villiger oxidation of the cyclic ketone into a lactone at the expense of NADH, whereas HLADH regenerates NADH, while producing the same lactone from the precursor diol substrate (Scheme 1).



Scheme 1. Fusion of FMO-E and HLADH applied in a convergent cascade reaction for the synthesis of γ-butyrolactone as a model lactone product.

In addition to cascade reactions, the use of unconventional media in biocatalysis has also been attracting much interest because the use of water as a reaction medium may have several limitations, such as 1) low solubility of hydrophobic substrates/products, 2) undesired side reactions, 3) tedious downstream processing, 4) enzyme inhibition issues by substrates/products dissolved in water, and 5) microbial contamination.[9] Although a two-liquid-phase system, typically with 50:50 ($v_{organic}/v_{aqueous}$) organic/aqueous phase, is an approach that removes some of these limitations, it is necessary to use higher volumetric ratios to achieve higher partitioning in the organic phase under equilibrium conditions.

Another alternative is the use of (predominantly) nonaqueous media. To use nonaqueous media, that is, solvent-free systems or organic solvents for redox catalysis, cofactor regeneration is still a challenge to be solved. During the 1980s and 1990s, extensive studies on the use of oxidoreductases under water-deficient conditions were reported by the research groups of Klibanov[9–11] and Adlercreutz,[12–16] It has been shown that substrate-coupled cofactor regeneration is possible in low-water media,[11,17,18] whereas enzyme-coupled cofactor regeneration is still not trivial because the nicotinamide cofactor (oxidized and reduced forms) needs to diffuse from one active site to the second one during the course of the reaction.

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One potential solution to make enzyme-coupled cofactor regeneration possible in low-water media is to fuse the two enzymes, so that the "cofactor travel distance" can be kept as short as possible; thus avoiding the degradation of nicotinamide cofactor by reaction media, for example, organic solvents.[19,20]

The aim of this study is to investigate whether fusing a monooxygenase and an alcohol dehydrogenase generates a bifunctional enzyme that can be used to catalyze a convergent cascade reaction in microaqueous media, by using predominantly organic solvents.

Design and construction of the fusion enzymes

First, the FMO-E-encoding gene from *Rhodococcus jostii* RHA1 and the HLADH isoenzyme E-encoding gene from *Equus caballus* were fused in both orientations in vector pET-28a(+). In this way, either of the enzymes being influenced by its fusion partner in one orientation could be identified. The two enzymes were fused by using a short glycine-rich peptide linker (SGSAAG); this has been found to be flexible in structure and typically does not influence the functioning of the fused enzymes.[21–24] The two resulting fusion enzymes were overexpressed in *Escherichia coli* BL21(DE3) and the activities of their cell-free extracts (CFEs) were measured and compared with those of the individual nonfused enzymes (Table 1). The FMO-E oxidation activity was analyzed by using 10 mM cyclobutanone as a substrate, whereas the HLADH oxidation activity was analyzed with 10 mM butane-1,4-diol.

The enzyme fused with HLADH at the C terminus retained much more activity than that of the reverse one, which showed almost no activity. This phenomenon is consistent with many other studies, in which short-chain dehydrogenases/reductases (SDRs) lose activity and/or stability if fused as a C-terminal fusion protein.[21,25] This might be caused by the perturbation of oligomers formed by these alcohol dehydrogenases (ADHs).[26] However, the relative activities of the two enzymes in the best performing fusion enzyme (FMO-E-HLADH) were also much lower (≈10%) than that of the individual enzymes. The fusion construct with FMO-E as the N-terminal fusion partner was then cloned into the pBAD vector and expressed in *E. coli* Top10, since FMO-E was originally expressed in this way.[27] The CFE of the *E. coli* Top10 cells expression fusion construct showed approximately three times higher activities for both enzymes (Table 1), perhaps because of higher enzyme expression (Figure S1 in the Supporting Information).

Enzyme purification and steady-state kinetic analysis

The fusion construct FMOE-HLADH cloned in the pBAD vector was then expressed in *E. coli* Top10 and purified by means of nickel affinity chromatography. The purification yield was approximately 40 mg of fusion enzyme per liter of culture broth after optimization of enzyme expression. The purified enzyme displayed a light-yellow color, which was indicative of binding of the flavin cofactor in FMO-E. From SDS-PAGE analysis of fusion enzyme purification, it was clear that proteolytic cleavage of a significant part of the purified fusion enzyme was observed (Figure 1), which could occur due to the sonication conditions and purification process. There were two protein bands detected in the purified fusion enzyme: the upper band (≈100 kDa) was the fusion enzyme (FMO-E-HLADH), whereas the lower one (≈64 kDa) was the FMO-E part of the fusion enzyme. Because FMO-E had the His-tag as an N-terminal tag, it could be purified even after the proteolytic cleavage of the fusion enzyme, whereas the HLADH part was lost during the purification process. To circumvent the proteolytic cleavage of fusion enzyme due to and during cell disruption, the French Press method (3×13000 psi on ice) was applied instead of sonication.

To verify the influence of fusion on the two enzymes, the kinetic parameters of the fusion enzyme and the individual enzymes were determined (Figure S4). Both enzymes showed approximately 60–70% activities (k_{cat} values), compared with the nonfused enzymes (Table 2), which may be caused by structur-

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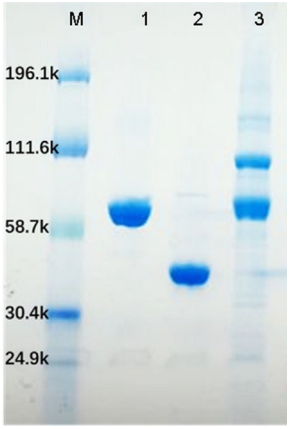

Figure 1. SDS-PAGE analysis of purified individual and fusion enzymes. M: marker; lane 1: FMO-E; lane 2: HLADH; lane 3: fusion enzyme FMO-E-HLADH.

Table 1. Activity assay of individual and fusion enzymes.

Enzyme	Vector	Host	FMO-E		HLADH	
			Specific activity [U g^{-1}][a]	Relative activity [%]	Specific activity [U g^{-1}][a]	Relative activity [%]
FMO-E	pBAD	*E. coli* Top10	296	100	–	–
HLADH	pET-28a(+)	*E. coli* BL21(DE3)	–	–	150	100
FMO-E-HLADH	pET-28a(+)	*E. coli* BL21(DE3)	40	14	16	11
HLADH-FMO-E	pET-28a(+)	*E. coli* BL21(DE3)	3	1	3	2
FMO-E-HLADH	pBAD	*E. coli* Top10	116	39	45	30

[a] The specific activity was calculated based on the protein concentration of the CFE.

Table 2. Kinetic constants of purified fusion and individual enzymes.

Enzyme	K_M [mM]	V_{max} [U mg^{-1}]	k_{cat} [s^{-1}]	K_i [mM]
FMO-E	2.4 ± 0.7	1.9 ± 0.2	2.0	–
fusion_FMO-E[a]	5.9 ± 1.6	1.3 ± 0.2	1.4	–
HLADH	2.4 ± 0.4	4.8 ± 0.2	3.2	2082 ± 755
fusion_HLADH[b]	2.1 ± 0.8	2.9 ± 0.5	1.9	6026 ± 275

[a] Activity assay applied for the fusion protein for the evaluation of the activity of FMO-E; [b] Activity assay applied for the fusion protein for the evaluation of the activity of HLADH. Fusion_FMO-E: FMO-E-HLADH assayed for FMO-E activity, Fusion_HLADH: FMO-E-HLADH assayed for HLADH activity.

al effects of bringing the two enzymes together. For FMO-E in the fusion enzyme, the affinity towards the substrate was affected by the fusion because the K_M value for cyclobutanone increased twofold. Conversely, HLADH in the fusion enzyme showed a somewhat lower K_M value; this indicated that the affinity was barely affected by the fusion. It was gratifying to note that the fusion enzyme displayed a higher K_i value towards butane-1,4-diol, which meant that the inhibition effect of the substrate on HLADH was alleviated. Overall, the fused enzyme largely retained the catalytic properties of the individual nonfused enzymes and was further investigated for its potential application in unconventional media.

Lyophilization of cell free extract of fusion enzyme

Because the aim of this study was to investigate the application of the fusion enzyme in unconventional media, lyophilization of the fusion enzyme could facilitate the use of enzymes in nonaqueous media, as well as shipping and storage in general.[28] From our previous study,[8] FMO-E was identified as having low thermo- and storage stability. Denaturation and deactivation of enzymes can take place upon freeze drying. However, additives can reduce aggregation/inactivation during the lyophilization or rehydration process, and hence, can compensate for the loss of essential water during lyophilization.[29] Therefore, we put effort into optimizing the lyophilization conditions of the fusion enzyme, especially for FMO-E part in the fused protein by using additives, namely, lyoprotectants. Sugars such as sucrose are widely used as lyoprotectants for the lyophilization process.[29–31] In addition to sugars, there are also other reported lyoprotectants, such as salts, reducing compounds, and amino acids.[30] Based on a literature search, we focused on sucrose and $MgSO_4$ because they have been shown to stabilize the lyophilization of many Baeyer–Villiger monooxygenases (BVMOs).[30]

To determine the protective effect of different concentrations of the selected additives, the CFE of the fusion enzyme was lyophilized with 10, 20, and 50 mg mL^{-1} sucrose, 10, 50, and 200 mM $MgSO_4$, and a combination of 20 mg mL^{-1} sucrose and 25 mM $MgSO_4$. Most of the additives resulted in a positive effect on the protection of the HLADH part, whereas only low concentrations of $MgSO_4$ as an additive could preserve the activities of FMO-E during lyophilization. The combination of sucrose and $MgSO_4$ did not show any additive effect, but only displayed a compromise of these two components. The best lyoprotectant for both FMO-E and HLADH was 50 mM $MgSO_4$. This slight improvement was significant enough to perform the lyophilization of the fusion enzyme with 50 mM $MgSO_4$ (Figure 2).

Employing fusion enzyme in micro-aqueous system

The lyophilized CFE of the fusion enzyme (in the presence of $MgSO_4$) was then applied to catalyze the model convergent

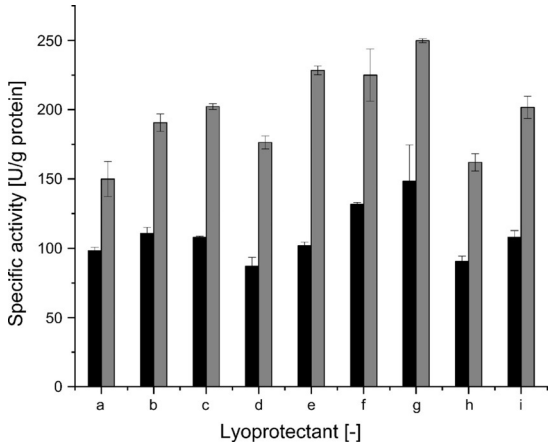

Figure 2. Influence of lyoprotectants on the lyophilization of fusion enzyme. A) Before lyophilization, B) no lyoprotectant, C) 10 mg mL^{-1} sucrose, D) 20 mg mL^{-1} sucrose, E) 50 mg mL^{-1} sucrose, F) 10 mM $MgSO_4$, G) 50 mM $MgSO_4$, G) 200 mM $MgSO_4$, and I) 20 mg mL^{-1} sucrose + 25 mM $MgSO_4$. The CFE of fusion enzymes was prepared in 10 mM pH 7.5 Tris·HCl buffer. Results are average values from experiments performed in duplicate. ■: FMO-E-HLADH assayed for FMO-E activity, ■: FMO-E-HLADH assayed for HLADH activity.

cascade reaction in a microaqueous system with 5% (*v/v*) buffer. Seven organic solvents, acetonitrile (ACN), isopropanol (IPA), tetrahydrofuran (THF), ethyl acetate (EtOAc), methyl *tert*-butyl ether (MTBE), cyclopentyl methyl ether (CPME), and *n*-heptane, were selected based on their different polarities (log *P*, logarithmic value of the octanol/water partition coefficient; Table S2) and because they have been applied in reactions catalyzed by dehydrogenases and monooxygenases.[32–36] Among these screened organic solvents, the two ether solvents, MTBE and CPME, gave the highest product concentration (Figure 3), whereby the log *P* value of MTBE was 1.0 and

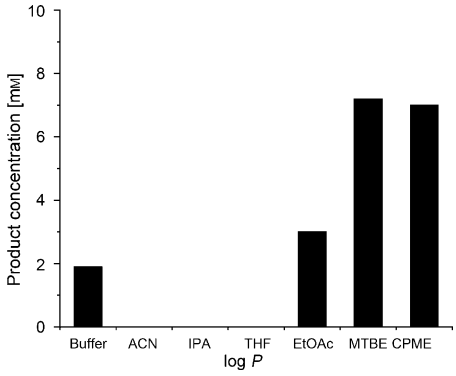

Figure 3. Screening of organic solvents for the convergent cascade reaction. Reaction conditions: *c*(cyclobutanone) = 20 mM, *c*(butane-1,4-diol) = 10 mM, 95% (*v/v*) organic solvent, 5% (*v/v*) external water (40 mM Tris·HCl, pH 7.5), 50 μL CFE, 20 °C, 900 rpm, and 48 h. log *P* (ACN) = −0.33, log *P* (IPA) = 0.05, log *P* (THF) = 0.53, log *P* (EtOAc) = 0.7, log *P* (MTBE) = 1.0, log *P* (CPME) = 1.41, and log *P* (*n*-heptane) = 4.47. 50 mM $MgSO_4$ was used as lyoprotectant for the preparation of the enzymes.

the log *P* value of CPME was 1.41. These results indicate that solvent functionality and structure are also important for enzyme deactivation. On the other hand, there was no product detected in the reaction systems containing ACN, IPA, and THF; these organic solvents with low log *P* values tended to strip the essential enzyme-bound water from the enzymes and resulted in less molecular flexibility for catalysis. The fused-enzyme-catalyzed control reaction in aqueous buffer generated about 3.5-fold less product (1.9 mM) than those synthesized in MTBE (7.2 mM) and CPME (7 mM) microaqueous systems (Figure 3). It is worth mentioning here that CPME currently refers to be an environmentally benign solvent. It has been applied in a microaqueous system with 10% (*v/v*) buffer for the reduction of β-carboline harmane and 1-methyl-3,4-dihydroisoquinoline into the corresponding amines catalyzed by an imine reductase (IRED).[37] MTBE has also been applied for the reduction of a series of ketones catalyzed by an ADH and promoted by the smart cosubstrate butane-1,4-diol in a microaqueous system with only 2.5% (*v/v*) buffer.[34] In that study, MTBE was selected from a series of organic solvents with log *P* values ranging from 1.0 to 5.6, owing to its high conversion, low boiling point, and good biocompatibility. It is commonly accepted that solvents with log *P* > 4 cause negligible inactivation of enzymes, whereas those solvents with log *P* < 2 are highly inactivating, and the effect of log *P* values between 2 and 4 is hard to predict.[38–40] However, in this study, log *P* cannot be the direct/only criterion to choose an organic solvent for enzymatic reactions.

The time courses of the lyophilized CFE of the fusion-enzyme-catalyzed model convergent cascade reaction in the microaqueous system of MTBE and CPME are shown in Figure 4. The two reaction systems showed almost the same

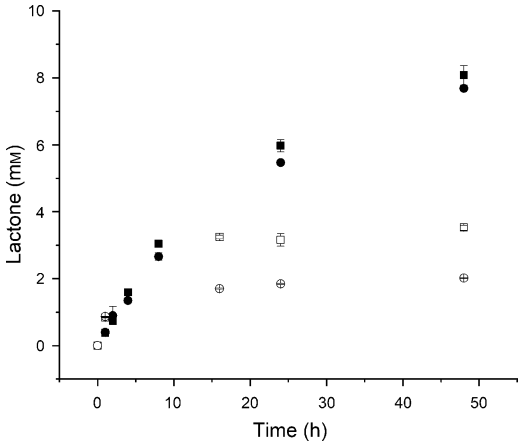

Figure 4. Time courses of the lyophilized fusion enzyme FMO-E-HLADH (closed symbols) and lyophilized unfused individual enzymes (open symbols) catalyzing a convergent cascade reaction in the microaqueous system of MTBE (squares) and CPME (circles). Reaction conditions: *c*(cyclobutanone) = 20 mM, *c*(butane-1,4-diol) = 10 mM, 95% (*v/v*) organic solvent, 5% (*v/v*) external water (40 mM Tris·HCl, pH 7.5), 1.5 mg mL^{-1} lyophilized fusion enzyme or 1.0 mg mL^{-1} lyophilized FMO-E and 0.5 mg mL^{-1} lyophilized HLADH, 20 °C, 900 rpm, and 48 h. Results are average values from experiments performed in triplicate. For the preparation of the enzymes, 50 mM $MgSO_4$ was used as a lyoprotectant.

progress curves and both resulted in about 8 mM product (≈27% analytical yield) after 48 h. Whereas if unfused enzymes, which were prepared under the same lyophilization conditions as those in the case of fused enzyme, were applied in the microaqueous media, the lactone concentration was 3.5 mM in MTBE and 2 mM in CPME (Figure 4). This can be attributed to the reduced travel distance of the cofactor with the fused enzyme. On the other hand, the effect of different organic media, that is, MTBE versus CPME, became significant for the unfused enzymes. It is worth mentioning here that these reactions were performed without external NAD^+ cofactor, and hence, it was only driven by the NAD^+ cofactors present in the CFE.

The model reaction was also performed under the same conditions with an additional 0.5 mM NAD^+ in the two systems. The addition of external NAD^+ resulted in about 20% increase in product formation in the case of fused enzyme (data not shown); this means that the cofactor is a minor limitation for the two reaction systems. Possibly one of the two enzymes was slowing or stopping, which would cause the other enzyme to stop, since both enzymes rely on the cofactor form (reduced or oxidized) that the other enzyme produces.

In summary, we have demonstrated the first application of a bifunctional fusion-enzyme-catalyzed convergent cascade in

microaqueous media. FMO-E and HLADH were combined with a glycine-rich linker as a bifunctional fusion enzyme. Sucrose and magnesium sulfate had positive effects on the lyophilization of the CFE containing the overexpressed fusion enzyme. The lyophilized CFE of fusion enzyme was applied for the convergent cascade reaction, consisting of cyclobutanone and butane-1,4-diol in microaqueous media with only 5% (*v*/*v*) aqueous buffer without any addition of external cofactor. MTBE and CPME were the best organic solvents in the microaqueous media for the fused protein FMO-E-HLADH. Overall, the cascade reaction catalyzed by fused oxidoreductase enzymes in predominantly organic media presented herein shows the high potential for these fragile enzymes to be employed under unconventional conditions.

Experimental Section

Chemicals, reagents, enzymes, and strains: Chemicals, media components, and reagents were purchased from Sigma–Aldrich, Carl Roth, Merck, Fluka, or Acros Organics and used without further purification. Nickel-NTA affinity resin was ordered from Expedeon (Cambridgeshire, UK) and BCA protein quantification kit (Pierce) was obtained from Thermo Scientific (Rockford, USA). The recombinant pET-28b(+) plasmid containing the HLADH gene was from Dr. Diederik Johannes Opperman (University of Free State, South Africa). Oligonucleotides were obtained from Sigma–Aldrich. T4 ligase and restriction enzyme BsaI were ordered from New England Biolabs. The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies. *E. coli* NEB 10-beta chemically competent cells were purchased from New England Biolabs and used as a host for cloning of the recombinant plasmids. Chemically competent *E. coli* BL21(DE3), *E. coli* Top10, cells were purchased from Invitrogen and used as a host for protein expression. Details on experimental protocols and analytics can be found in the Supporting Information.

Employing the fusion enzyme in a convergent cascade in microaqueous media: Lyophilized CFE of fusion enzyme (from 2 mL CFE) was redissolved in doubly distilled H_2O (500 μL). Cyclobutanone (20 mM, 5.95 μL) and butane-1,4-diol (10 mM, 3.6 μL) were prepared in organic solvents (4 mL) to form substrate stocks. The reactions were started by adding the enzyme solution (50 μL) to substrate stocks (950 μL). Therefore, the starting concentrations were 20 mM cyclobutanone, 10 mM butane-1,4-diol, 95% (*v*/*v*) organic solvent, and 5% (*v*/*v*) aqueous buffer. The total reaction volume was 1.0 mL and the reaction mixtures were kept at 20 °C and 900 rpm. Aliquots of samples (50 μL) from the organic phases were taken at definite time intervals and mixed with EtOAc (250 μL), followed by vigorous mixing and drying over anhydrous $MgSO_4$. The samples were then analyzed by means of GC. If phase separation occurred, the EtOAc phase was taken for GC analysis.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · domino reactions · enzymes · fusion enzymes · solvent effects

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