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Development of a simplified purification method for a novel formaldehyde dismutase variant from *Pseudomonas putida* J3

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ABSTRACT

Formaldehyde dismutase (FDM) is a very interesting enzyme, due to the fact that it comprises an internal cofactor regeneration mechanism. The FDM, therefore, is able to catalyze redox reactions independent of exogenous cofactor addition, rendering the enzyme powerful for industrial applications. Currently, only one enzyme of this type has been characterized enzymatically. Furthermore, only one additional DNA-sequence with high homology to FDM has been published. In this work, we identified a new variant of a formaldehyde dismutase gene (*fdm*) in the *Pseudomonas putida* J3 strain. To isolate and characterize the enzyme, we developed a simplified method for its purification. This purification is based on a C-terminal 6xHis-tag, which enables functional expression of the enzyme in *E. coli* and a one-step purification method. In addition, we tested several expression systems for optimal yields and combined this with co-expression of the chaperonins GroESL. Using this simplified and rapid method, we are now able to produce sufficient material in reproducible quality and quantity for application tests with the enzyme. The newly identified enzyme will be applied in a redox cascade for biomethanol production from biogas and shows potential for further industrial biotransformation with integrated cofactor recycling.

1. Introduction

Oxidoreductases are strongly underrepresented in industrial biotransformation due to their need of cofactors as electron carriers and the consequently required external cofactor recycling. One interesting oxidoreductase with integrated cofactor recycling is formaldehyde dismutase (FDM) which catalyzes the dismutation of formaldehyde in a ping-pong mechanism (Fig. 1). Yielding formic acid and methanol as products from a successive oxidation and a reduction reaction, respectively, FDM does not need external cofactor recycling strategies. Exploiting this beneficial trait, FDM from *Pseudomonas putida* F61 has been applied in several enzyme cascades with differing purposes. First, FDM was used for formic acid production from methanol together with alcohol oxidase (AOX) and catalase (Mizuno and Imada, 1986). In another attempt, a regeneration system for reduced NAD-cofactors was developed by combining FDM, alcohol dehydrogenase (ADH) and formate dehydrogenase (FDH) (Kara et al., 2015). Furthermore, an *in situ* hydrogen peroxide generation system was established using FDM and AOX (Ni et al., 2016); thereby, a peroxygenase-catalyzed stereoselective oxyfunctionalization was enabled. To evaluate further applications for FDM it would be desirable to rapidly produce the enzyme in a simple and efficient way.

Only two variants of the enzyme (strain *P. putida* F61 and *P. putida* J3) have been described previously (Kato et al., 1983; Rodewyk, 1998). The sequence of F61 has been published by Yanase et al. as well as a functional characterization of the enzyme (Yanase et al., 1995, 2002). FDM from J3 has been characterized on the enzyme level only until now (Rodewyk, 1998). Furthermore, only one additional DNA sequence has been reported so far (Jin et al., 2016). This apparent limitation in occurrence of this enzyme may have hampered an efficient screening for industrial applications of this enzyme. Also the current purification methods require high expertise and considerable amount of time. Tetrameric FDM has been purified previously directly from *P. putida* F61 (Kato et al., 1983) and was also expressed in *Escherichia coli* by Yanase et al.

Abbreviations: ADH, alcohol dehydrogenase; AOX, alcohol oxidase; CV, column volume; FDH, formate dehydrogenase; FDM, formaldehyde dismutase; IMAC, immobilized metal affinity chromatography; MBP, maltose binding protein; MWCO, molecular weight cut-off.

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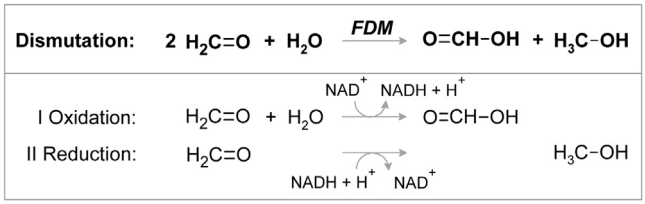

Fig. 1. Dismutation reaction of formaldehyde catalyzed by formaldehyde dismutase showing its cofactor recycling mechanism. Half reaction I shows the oxidation of formaldehyde to formic acid and half reaction II shows the reduction of formaldehyde to methanol with respective reduction/oxidation of the tightly bound cofactor NAD.

using lac, tac and T7 promoter systems (Yanase et al., 1995, 2002). In these publications classical purification technologies were used, including the combination of several precipitation and chromatography steps.

To simply the purification of FDM we developed a one-step purification method, based on a C-terminal affinity tag. We tested several expression systems for optimal yields and combined this with co-expression of the chaperonins GroESL as described previously (Yanase et al., 2002). Among our *E. coli* expression systems the rhamnose promoter system yielded the best results. Benefits of the rhamnose system have been described as high expression levels, high solubility of overproduced proteins, very tight control and high plasmid stability due to the encoded cer-sequence on the expression plasmid (Wilms et al., 2001; Wegerer et al., 2008).

Using this simplified and rapid method, we are now able to produce sufficient material in reproducible quality and quantity for application tests with the enzyme. The newly identified enzyme is currently applied in a redox cascade for biomethanol production from biogas and shows potential for further industrial biotransformation with integrated cofactor recycling.

2. Materials and methods

2.1. Materials

All DNA oligonucleotides were synthesized by Eurofins MWG Operon, Germany. L-Rhamnose was kindly provided by Suedzucker (Germany). Formaldehyde detection was done by the cuvette test LCK 325 (HACH LANGE GmbH, Germany). All standard reagents were of analytical grade.

2.2. Bacterial strains and plasmids

Escherichia coli ER2523 and plasmid pMAL-p5X were purchased from NEB (Germany), *E. coli* JM109 from Promega Corporation (USA) and pUC118 from Takara Bio Inc. (Japan). The rhamnose system comprised the rhamnose-deficient host strain *E. coli* BW3110 (Wilms et al., 2001) and expression plasmid pJOE5940.1 carrying the rhamnose promoter P_{rhaBAD} (all strains see Table 1)). All plasmids were selected for with ampicillin (100 μg mg^{-1}) except for pKY206 which was selected for with tetracyclin (12.5 μg mL^{-1}). The *Pseudomonas putida* strain J3 was isolated from an enrichment culture from sewage sludge at Fraunhofer-Institute for Interfacial Engineering and Biotechnology IGB Stuttgart (Germany) (Rodewyk, 1998) and classified by DSMZ (German Collection of Microorganisms and Cell Cultures Braunschweig, Germany).

2.3. DNA and strain engineering

For isolation of genomic DNA, *P. putida* J3 was grown on liquid medium (10 g L^{-1} peptone, 10 g L^{-1} meat extract and 5 g L^{-1} NaCl, pH 7.0–7.2) at 30 °C. Genomic DNA from *P. putida* J3 was extracted chemo-mechanically and used as template for the amplification of the gene encoding formaldehyde dismutase (*fdm*).

For cloning, *E. coli* strains were cultured at 37 °C on LB medium (10 g L^{-1} bacto peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.0) with 100 μg mL^{-1} ampicillin and 12.5 μg mL^{-1} tetracycline where appropriate.

Based on sequence information of the *P. putida* F61 gene locus, primers were chosen for *P. putida* J3 in the 5′- and 3′ non-coding regions (5′-CTTGACAGGGCTTTGTTCC-3′ and 5′-TAGTGGGGCAGGCTTTC-3′). Subcloning was done to introduce the *fdm* J3 gene into pUC118 (BamHI/PstI), pMAL-p5X-ΔmalE (BamHI/PstI) and pJOE5940.1 (NdeI/BsrGI) (strains and plasmids see Table 1). The pMAL-p5X-ΔmalE plasmid was the result of an *Eco*53kI/*Mfe*I digest of pMAL-p5X to eliminate the *malE* gene. The C-terminal 6xHis-tag was attached to *fdm* J3 on pJOE5940.1 via the reverse PCR primer (5′-TGTACATCAATGATGATGATGATGTTTATTCTTCAACATGC-3′). For chaperone co-expression, plasmid pKY206 (Ito and Akiyama, 1991) was transformed to *E. coli* BW3110/pJOE-J3 or -J3-6xHis.

2.4. Formaldehyde dismutase expression in Escherichia coli

Preculture I (5 mL of LB-medium + respective selective pressure in test tubes) was inoculated with cell material from agar plates and grown at 37 °C for 6–8 h. Preculture II (20 mL of LB-medium + respective selective pressure in baffled shaking flasks) was inoculated 1:100 from preculture I and grown for 16–18 h at 30 °C. Main cultures were started from preculture II by diluting to OD_{500nm} of 0.13. Induction by the respective inducer was done either in early exponential phase or in transition state to stationary phase (see results). Cells were harvested in late stationary phase (24 h) or as indicated in the results section.

2.5. Preparation of protein extracts and precipitate fractions

E. coli cells were harvested by centrifugation at 4 °C and 4,696 × *g* for 10 min, washed in 0.9% NaCl and resuspended in 100 mM Na_2HPO_4 (pH 7), treated 4 times with indirect ultrasound for 30 s with 100% amplitude and centrifuged at 16,060 × *g*, 4 °C for 5 min. The supernatant (protein extract) was used in SDS-PAGE analysis and activity assay. Insoluble precipitate fractions were resuspended in 6 M urea, the volume being the same as the original cell suspension before cell disruption. These suspensions were incubated at room temperature for 1 h and centrifuged at 16,060 × *g*. The supernatant containing re-dissolved insoluble aggregates was analyzed by SDS-PAGE.

For FDM purification, cells were disrupted by a French press (EmulsiFlex-B15, Avestin, Germany). The cell-free extract was obtained as the supernatant after centrifugation (4,696 × *g*, 4 °C, 15 min) and analyzed as described above.

2.6. Protein analysis

Total protein concentration was measured with the Bio-Rad Protein Assay (Bradford) with bovine serum albumin as standard according to the manufacturer's protocol (Bio-Rad Laboratories GmbH, Germany). For SDS-PAGE, 10 μg of total protein extracts or 5 μg of purified protein was loaded; for the redissolved precipitate fraction, the same volume was loaded as for the corresponding protein extract.

2.7. Activity assay

FDM activity was assayed via determination of formaldehyde degradation within 5 min reaction time at room temperature using the formaldehyde test LCK325 (HACH LANGE GmbH, Germany). The