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Research paper

Identification and optimization of a novel thermo- and solvent stable ketol-acid reductoisomerase for cell free isobutanol biosynthesis

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ABSTRACT

Due to its enhanced energy content and hydrophobicity, isobutanol is flagged as a next generation biofuel and chemical building block. For cellular and cell-free isobutanol production, NADH dependent (over NADPH dependent) enzyme systems are desired. To improve cell-free isobutanol processes, we characterized and catalytically optimized a NADH dependent, thermo- and solvent stable ketol-acid reductoisomerase (KARI) derived from the bacterium *Meiothermus ruber* (Mr). The wild type Mr-KARI has the most temperature tolerant KARI specific activity reported to date. The KARI screening procedure developed in this study allows accelerated molecular optimization. Thus, a KARI variant with a 350% improved activity and enhanced NADH cofactor specificity was identified. Other KARI variants gave insights into Mr-KARI structure–function relationships.

1. Introduction

Ketol-acid reductoisomerase (KARI, EC 1.1.1.86) catalyzes the unusual two-step conversion of 2-acetolactate into 2,3-dihydroxyisovalerate via an initiating alkyl migration and a following NAD(P)H dependent reduction [1]. The Mg^{2+} dependent KARI activity is present in plants, fungi and microorganisms [1]. Recently, the KARI dependent part of branched-chain amino acid biosynthesis has attracted great interest in both sustainable agrochemical- and biofuel research. Especially isobutanol is flagged as a next generation biofuel and sustainable chemical building block due to its enhanced energy content and hydrophobicity compared to ethanol. The metabolic conversion of the 2-keto acid to the target alcohols involves the host specific oxidoreductase activities of KARI and an alcohol dehydrogenase (ADH).

Recently, *E. coli* specific KARI has been successfully engineered to accept NADH to significantly improve isobutanol yields [2]. A generally applicable method for modification of the KARI superfamily has been reported by Brinkmann-Chen et al. [3]. Although, significant improvements in isobutanol biosynthesis have been implemented at a molecular level, current fermentative processes have a product ceiling of 2% (v/v) due to toxicity effects and non-productive metabolic side reactions [4].

More recently, a cell-free isobutanol production system based on a designed enzyme cascade has been reported [5], which holds the promise of higher isobutanol titers and simplified product recovery. This redox–neutral reaction cascades utilizes NAD^+ as the universal cofactor and operates at elevated temperatures (i.e. 50 °C) and solvent concentrations. These process parameters enable isobutanol titers in excess of those obtainable with cellular systems. Further, since process conditions and substrate/intermediate specificities are strictly controlled, unproductive side reactions are eliminated, which allows a targeted conversion of sugars into isobutanol. The final process configuration of the cell-free isobutanol production system is only limited by the operational parameters of the individual enzyme components [5]. Key to implementation of this cell-free reaction cascade was the exchange of NADPH dependent oxidoreductase activities with equivalents accepting NADH as the cofactor. While aldehyde and alcohol dehydrogenases could be substituted by reported NADH dependent alternatives [6,7], the search for a NADH dependent KARI activity remained challenging, especially as process stability at high temperature and high solvent concentrations were additional enzyme selection criteria. None of the reported KARI enzyme activities met the desired process parameters with respect to temperature tolerance, while no data was available for solvent tolerance.

Abbreviations: KARI, ketol-acid reductoisomerase; Mr, *Meiothermus ruber*; Cg, *Corynebacterium glutamicum*; Ss, *Sulfolobus solfataricus*; Se, *Slackia exigua*; Ec, *Escherichia coli*; τ, half-life; ADH, alcohol dehydrogenase; WT, wild-type; CV, coefficient of variance; Z′, dimensionless parameter for the quality of an assay itself; Z, dimensionless parameter for the quality of an array of tested variants.

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In this study, we present a new NADH dependent KARI activity isolated from the bacterium *Meiothermus ruber* DSM 1279 (Mr-KARI). This enzyme features an excellent catalytic performance together with pronounced thermal- and solvent stabilities. The ideal combination of these process relevant parameters, allowed successful incorporation of the wild type Mr-KARI activity in our designed cell-free isobutanol production system [5].

Since the catalytic performance of the wild type KARI was a limiting factor in achieving even higher isobutanol yields, we addressed this issue by combining directed evolution with targeted genetic engineering strategies. Here we report on the detailed characterization of wild type KARI and present a new KARI directed *in-vivo* screening methodology. Application of this new methodology allowed identification of various KARI variants with enhanced catalytic efficiency. The variant T84S could be purified to homogeneity and was thoroughly characterized.

2. Materials and methods

2.1. Reagents and kits

Restriction enzymes, T4 DNA ligase, T4 Kinase, Shrimp alkaline phosphatase, Phusion polymerase and desoxynucleotides were purchased from Thermo Scientific (Ulm, Germany). Desoxyribonuclease I from bovine pancreas was from Serva Electrophoresis (Heidelberg, Germany). All enzymes were used according to the manufacturers' recommendations, applying the provided buffer solutions. Oligonucleotides were ordered from Eurofins MWG Operon (Ebersberg, Germany). All chemicals were, unless otherwise stated, purchased in analytical grade from Sigma-Aldrich (München, Germany), Carl Roth (Karlsruhe, Germany), Serva Electrophoresis and AppliChem (Darmstadt, Germany). Plasmids were purified applying the GeneJET Plasmid Miniprep Kit (Thermo Scientific), PCR products and enzymatically manipulated DNA were purified via the innuPREP DOUBLEpure Kit (Analytik Jena, Germany).

2.2. Strains and media

Meiothermus ruber DSM 1279 (DSMZ, Braunschweig, Germany) was grown aerobically at 50 °C in *Thermus ruber* medium containing trypton (5 g L^{-1}), yeast extract (1 g L^{-1}) and soluble starch (1 g L^{-1}), adjusted to pH 8. *E. coli* Rosetta cells (F- ompT hsdSB(rB-mB-) gal dcm (DE3) pLysSRARE (CamR)) were purchased from Merck (Darmstadt, Germany) and were grown in TB medium for protein expression. The KARI knockout strain *E. coli* JW3747 (F-, Δ(araD-araB)567, ΔlacZ4787(:rrnB-3), λ-, rph-1, ΔilvC725:kan, Δ(rhaD-rhaB)568, hsdR514) was obtained from the Keio collection [8]. This strain was grown in M9 minimal medium. *E. coli* XL1-Blue cells (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZDM15 Tn10 (Tetr)]) from Stratagene (Waldbronn, Germany) were grown in LB medium. All media were supplemented with kanamycin (30 μg ml^{-1}), TB additionally with chloramphenicol (34 μg ml^{-1}).

2.3. Sequence alignment and structural modeling

KARIs have been observed in several pro− and eukaryotes. A number of gene and protein sequences may be found in various databases, although not all are characterized. For the amino acid alignment we chose only bacterial sequences retrieved from the UniProt database [9]. Clustal Omega [10–12] was used to conduct the sequence alignment and ESPript [13] for further editing. Examination of structure–function relationships based on a modeled Mr-KARI structure conducted by the Phyre^2 server [14]. After primary sequence alignment the published crystal structure of *Pseudomonas aeruginosa* (PDB 1NP3) KARI was used as a template to compute the Mr-KARI structural features. Subsequently, respective KARI ligands Mg^{2+}, 2,3-dihydroxy-3-methylvalerate and $NADP^+$ were adopted from the spinach scaffold (PDB 1YVE and 1QMG).

2.4. DNA isolation and cloning

The plasmid pET28a (Merck) was pretreated as described by Guterl et al. [5]. Genomic DNA from *Meiothermus ruber* DSM 1279 was isolated as described by Saha [15]. The gene *mrub_1907* was amplified from genomic DNA by PCR with the phosphorylated primers listed in Table 1. PCR fragments were ligated into pCBR via the BsmBI restriction site. The obtained pCBR-Mr-KARI-CHis plasmid was transformed in *E. coli* as described elsewhere [16]. DNA sequencing validated all cloning procedures.

2.5. Protein expression and purification

For protein expression, transformed cells were cultivated in a shaking flask at 37 °C in TB medium supplemented with kanamycin (30 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). The cells were induced with 1 mM IPTG at OD_{600} 0.5–0.8 and afterwards incubated at 20 °C for 20 h.

The basal expression of Mr-KARI in the knockout strain JW3747 was conducted in M9 minimal medium supplemented with 0.4% (w/v) glucose and kanamycin (30 μg ml^{-1}) as indicated [16]. The culture was inoculated with transformed cells and cultivated at 37 °C overnight. Cultures were subsequently harvested and frozen at −20 °C until further use.

For cell disruption the cell pellets were resuspended in binding buffer (50 mM HEPES pH 8, 20 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Tween 20), supplemented with DNAse (10 mg ml^{-1}). The cells were lysed with an Avestin EmulsiFlex-B15 homogenizer. Debris was removed by centrifugation at 20,000 × g and 4 °C for 30 min. The supernatant was loaded onto Ni-NTA columns and washed with 5 column volumes of binding buffer. His-tagged Mr-KARI was eluted in one step with two column volumes of elution buffer (50 mM HEPES pH 8, 500 mM imidazole, 10% glycerol, 0.1% Tween 20). All fractions were analyzed by 12% SDS-PAGE.

The purified enzyme was subsequently desalted via HiPrep 26/10 Desalting-column (GE Healthcare Europe; Freiburg, Germany) in either 20 mM ammonium carbonate for lyophilization or in 50 mM HEPES pH 7.5 plus 10% glycerol for storing at −80 °C.

Protein contents were quantified spectrophotometrically by measuring the absorbance at 215 and 225 nm as indicated [17–19].

2.6. Enzyme characterization

All assays were performed in microtiter plate format using an Enspire 2 (Perkin Elmer; Rodgau, Germany). The reaction was initiated by addition of 180 μl assay mixture to 20 μl enzyme solution. Assay mixtures were preincubated in a thermomixer for accurate temperature control. The pH was adjusted to the corresponding temperature. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per minute.

Table 1
Oligonucleotides.

Primer	Sequence (5′ − 3′)
Mr-KARI fwd	CAGCAACGTCTCGCATATGAAGATTTACTACGACCAGGACGCAG
Mr-KARI rev	GCTACCGACCTCTTCCTTCGTGAAC