Enzyme and Microbial Technology 47 (2010) 140–146

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Directed evolution of *Alcaligenes faecalis* nitrilase

Ulrike Schreiner[a], Bianca Hecher[a], Sascha Obrowsky[a], Kerstin Waich[a], Norbert Klempier[b], Georg Steinkellner[a,c], Karl Gruber[c], J. David Rozzell[d,1], Anton Glieder[a], Margit Winkler[a,*]

[a] *ACIB GmbH c/o Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria*
[b] *Institute of Organic Chemistry, Graz University of Technology, Stremayrgasse 16/2, 8010 Graz, Austria*
[c] *Institute of Molecular Biosciences, University of Graz, Humboldtstraße 50/3, 8010 Graz, Austria*
[d] *Codexis Inc. 200 Penobscot Drive, Redwood City, CA 94063, USA*

ARTICLE INFO

Article history:
Received 16 February 2010
Received in revised form 27 May 2010
Accepted 27 May 2010

Keywords:
Enzyme catalysis
Nitrilase
Directed evolution
Mutation
pH stability

ABSTRACT

Alcaligenes faecalis nitrilase (NIT*Af*) was engineered in a directed evolution approach towards increased activity at its optimal pH as well as improved fitness at low pH values. Error prone PCR in combination with recombination of beneficial mutations resulted in a variant with increased specific activity for 2-phenylpropionitrile at pH 7.5. In addition, a new nitrilase variant (pHNIT45) was developed that is catalytically active at pH values as low as pH 4.5. Within 10 min this mutant fully hydrolyzes the base labile substrate (*R*)-2-Cl-mandelonitrile (10 mM) to give the product (*R*)-2-Cl-mandelic acid (conversion 100%, *ee* > 99%) with full retention of enantiopurity.

1. Introduction

Nitrilases (EC 3.5.5.X) are enzymes which hydrolyze nitriles to the corresponding carboxylic acids and ammonia [1]. In contrast to conventional chemical methods the enzyme-catalyzed hydrolysis is carried out at ambient temperature, does not require strongly acidic or basic reaction conditions and, depending on the enzyme, can be enantioselective. These mild reaction conditions are especially advantageous for nitriles that carry acid- or alkaline-labile functionalities [2]. Even though there are some biocatalytic processes on industrial scale—the most prominent being the hydrolysis of racemic mandelonitrile to (*R*)-mandelic acid catalyzed by nitrilases from several *Alcaligenes* strains [3]—applications as versatile biocatalysts are limited due to low stability, selectivity or specific activity of nitrilases [4]. The lack of a crystal structure of any closely related enzyme prohibited the creation of precise nitrilase 3-D models and thereby rational protein design as a tool for targeted nitrilase engineering. The alternative to amend the features of certain nitrilases is directed evolution, which has been demonstrated for five different nitrilases [5–10].

The nitrilase from *Alcaligenes faecalis* JM3 (swissprot: P20960) was characterized for the first time in 1990 and acts preferentially on arylacetonitriles [11]. It was shown that the gene coding for this nitrilase is identical to the nitrilase gene of *A. faecalis* ATCC8750 [12]. Surprisingly, a nitrilase from *Pseudomonas putida* was found to be identical to the sequence of the *A. faecalis* JM3 nitrilase [13]. The nitrilase from *Pseudomonas fluorescens* EBC191 showed 48% identity. In contrast to other nitrilases such as the nitrilase Nit2 from *Arabidopsis thaliana* [14] these nitrilases tolerate substrates with substituents in the alpha position of the nitrile, as for example in mandelonitrile (benzaldehyde cyanohydrin) and 2-phenylpropionitrile **1**. Such α-branched arylacetonitriles and their derivatives are important precursors for the production of many pharmaceutical and agricultural products [15]. Racemic mandelonitrile and 2-phenylpropionitrile were reported to be converted more efficiently by the nitrilase of *P. fluorescens* EBC191 compared to the *Alcaligenes* nitrilase [16]. However, it generally showed lower enantioselectivity and preferentially formed the (*S*)-acid. The *Alcaligenes* nitrilase, in contrast, exhibited excellent enantioselectivity towards the (*R*)-enantiomer of mandelonitrile [16]. Another advantageous characteristic of the *Alcaligenes* nitrilase is the formation of negligible or very low amounts of amide as a side product. Kiziak et al. found that on one hand the nature of the C-terminal region and on the other hand two amino acids (L168 and S169) that are very close to the catalytic cysteine, are responsible for reduced amide formation compared to the *P. fluorescens* EBC191 nitrilase [12,16].

* Corresponding author. Tel.: +43 316 873 9333; fax: +43 316 873 9302.
E-mail address: margit.winkler@acib.at (M. Winkler).
[1] Current address: Solidus Biosciences, 409 Illinois Street, Suite 2073, San Francisco, CA 94158, USA.

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doi:10.1016/j.enzmictec.2010.05.012

We aspired to improve the fitness of the *A. faecalis* nitrilase (swissprot: P20960) for the hydrolysis of sterically hindered substrates by directed evolution. 2-Phenylpropionitrile (**1**) was used as a model substrate to evaluate enzyme variants after a first round of mutagenesis. The screening for higher activity was performed at the pH optimum [17] of the wild type enzyme (pH 7.5) as well as at low pH (pH 4.5), for application of base labile α-branched substrates such as cyanohydrins [18]. The intrinsic stability of nitrilases under acidic conditions is generally low. At pH values <5 all known nitrilases are rapidly inactivated [18]. We have therefore especially focused on the development of a nitrilase with high activity for the sterically hindered 2-phenyl substituted nitriles at low pH. Enzyme preparations such as whole cell biocatalysts [18,19] immobilized enzymes or CLEAs [20,21] are envisaged to additionally improve the operational stability of nitrilases for low pH applications.

2. Materials and methods

2.1. Gene mutagenesis and library construction

The expression plasmid BT5291 contained the wild type gene nit*Af* with an additional C-terminal HIS-tag and a stabilizing glycine after the start codon. Error prone polymerase chain reaction (PCR) was carried out on this template with *Taq* polymerase.

Primers were purchased from Invitrogen, Carlsbad, CA. The reaction mixture (50 μL) contained *Taq* Polymerase (0.3 μL, 5 U μL^{-1}, Fermentas, Burlington, Canada), template BT5291 (1.25 μL, 8 ng μL^{-1}), dNTP mix (10 μL, 2 mM), forward primer P05-346 (5′-GGCACTCGACCGGAATTATC-3′, 2 μL, 10 pmol μL^{-1}), reverse primer P05-348 (5′-ACGGCGCTATTCAGATCCTC-3′, 2 μL, 10 pmol μL^{-1}), *Taq* buffer with $(NH_4)_2SO_4$ (5 μL, 10×), $MgCl_2$ (14 μL, 25 mM) diluted with doubly distilled H_2O to 50 μL. For the first mutant library $MnCl_2$ (0.5 μL, 20 mM) was added. The PCR was conducted on a Gene Amp System 2400 thermo cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 2 min, 30 cycles of 30 s at 95 °C, 30 s at 63 °C and 80 s at 72 °C and 10 min at 72 °C for the final elongation step. The PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and digested with *Nco*I and *Bam*HI as well as the vector pTrc-Kan.

The digested fragments were separated on a 0.8% (w/v) agarose gel and the DNA isolated from the excised gel pieces using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). Prior to ligation, the digested vector DNA was dephosphorylated with SAP (Shrimp Alkaline Phosphatase, 1 U μL^{-1}, Fermentas, Burlington, Canada) according to the suggested protocol and then purified with the QIAquick® PCR Purification Kit. The ligation was performed with T4 DNA ligase (5 U μL^{-1}, Fermentas, Burlington, Canada) at 16 °C over night. After desalting (0.025 μm nitrocellulose membrane filters, Millipore, Billerica, MA), an aliquot of the ligation mixture (5 μL) was transformed in 80 μL electrocompetent *Escherichia coli* TOP 10 F′ cells (Invitrogen, Carlsbad, CA) and plated on LB agar plates containing kanamycin (25 μg mL^{-1}).

Single colonies (10,000 transformants) were picked with a picking robot QPixII (Genetix, New Milton, UK) into 384 square well plates containing TB medium (50 μL) supplemented with kanamycin (50 μg mL^{-1}) per well. The plates were incubated for 24 h at 37 °C in a covered, water containing glass container without agitation to reduce media evaporation. A mixture of TB-kanamycin (50 μg mL^{-1}) medium with 40% (v/v) of glycerol was added per well (30 μL) resulting in a final glycerol concentration of 15%. The 384 well plates were covered with an aluminum seal and stored at −80 °C.

The following rounds of random mutagenesis were performed with Mutazyme® II DNA polymerase from the GeneMorph® Random Mutagenesis Kit (Stratagene, Foster City, CA) in order to avoid the mutation bias of Taq-polymerase for certain positions. The number of colonies picked for the second round was 4750, for the third round 6630 and for the fourth round 3170. The random mutagenesis library which was screened at pH 4.5 consisted of 3430 colonies. The mutated genes were generated as megaprimer using the following reaction mixture (50 μL): plasmid DNA of each starting clone (500 ng), dNTP mix (1 μL, 40 mM), Mutazyme® II reaction buffer (5 μL, 10×), forward primer P06-746 5′-ATGGGTCAGACTCGTAAAATC-3′ (2 μL, 10 pmol μL^{-1}), reverse primer P06-747 5′-TTAGTGATGGTGATGGTGATG-3′ (2 μL, 10 pmol μL^{-1}), Mutazyme® II DNA polymerase (1 μL, 2.5 U μL^{-1}), diluted with doubly distilled H_2O to 50 μL. The following PCR program was used: 95 °C for 2 min, 25 cycles of 30 s at 95 °C, 30 s at 60 °C and 70 s at 72 °C and 10 min at 72 °C for the final elongation step.

The PCR product was separated from the template plasmid DNA on a 0.8% (w/v) agarose gel, the desired DNA band excised and the DNA isolated using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). The purified megaprimers were extended in a second PCR using 100 ng of the same plasmid DNA as template as in the first PCR, 15 μL of purified megaprimer (100 ng μL^{-1}), dNTP mix (5 μL, 2 mM), DMSO (1.6 μL, 100%), *Pfu*Ultra™ HF reaction buffer (5 μL, 10×), *Pfu*Ultra™ HF DNA polymerase (1 μL, 2.5 U μL^{-1}) and diluted with doubly distilled H_2O to 50 μL. The PCR was conducted under the following conditions: 95 °C for 2 min, 25 cycles of 50 s at 95 °C, 60 s at 60 °C and 15 min at 68 °C. After adding *Dpn*I (2 μL, 10 U μL^{-1}) the PCR reaction was incubated for 2 h at 37 °C followed by an inactivation step at 70 °C for 10 min. For transformation, picking and library conservation see above.

2.2. Enzyme screening (Berthelot assay)

After thawing the glycerol stocks of the mutant libraries, four 96 well flat bottom plates were inoculated from one 384 well plate using a replicator. The cells were grown in 150 μL TB-kanamycin (50 μg mL^{-1}/well) at 28 °C over night. For the main culture, 96 well V-bottom plates with 150 μL TB-kanamycin (50 μg mL^{-1}) containing isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) were used and were incubated at 28 °C for 20 h after inoculation. Cells were harvested at 4 °C and 1552 × *g* for 12 min. The supernatant was discarded and the pellets were washed with distilled water (100 μL per well). After centrifugation at 4 °C and 2113 × *g* for 15 min and discarding the supernatant, the pellets were frozen at −80 °C for at least 1 h. Cell lysis was performed using lysis buffer (40 μL/well; 1.0 mg mL^{-1} lysozyme, 0.1% (w/v) Triton X-100 and 50 mM potassium phosphate buffer pH 7.5). The resuspended cells were incubated at 28 °C for 1 h. Depending on the enzyme activity, the cell lysate was diluted with potassium phosphate buffer (50 μL or 100 μL, 50 mM, pH 7.5). The supernatant containing the enzyme was obtained by removing the cell debris by centrifugation at 4 °C and 2113 × *g* for 15 min. 5 μl (10 μl for screening at pH 4.5) of the clarified lysate were transferred into a new 96 well flat bottom plate and used for the conversion of **1**. The reaction solution (45 μL, 50 mM potassium phosphate buffer pH 7.5, or 50 mM sodium citrate buffer pH 4.5, **1** 10 mM, 2% methanol) was added per well. After an appropriate reaction time, the hydrolysis was stopped by adding sodiumphenolate (50 μL, 0.4 M) and sodium nitroprusside dihydrate (50 μL, 0.02% (w/v)). The blue colored indophenol developed after adding sodium hypochlorite (50 μL, 0.5% (v/v)). The plates were sealed with an aluminum tape and incubated at 95 °C for 10 min. After cooling to room temperature, the absorbance was measured at 640 nm in a platereader. The best hits were analyzed in two consecutive rescreens using the Berthelot assay and mutants with the highest improvement were cultivated in a fermenter followed by protein purification.

2.3. Protein expression and purification

The wild type NIT*Af* and the mutants pHNIT45 as well as 9/H6 were cultivated in 4 L fermentation media, consisting of the following: 25 g L^{-1} yeast extract, 13 g L^{-1} ammonium chloride, 9 g L^{-1} potassium dihydrogen phosphate, 5 g L^{-1} disodium hydrogen phosphate, 0.08 g L^{-1} ferrous citrate, 0.05 g L^{-1} zinc sulfate heptahydrate, 15 g L^{-1} glucose, 1.3 g L^{-1} magnesium sulfate and 0.04 g L^{-1} thiamin. An over night culture was used to inoculate a pre culture in 500 mL TB medium (12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 0.17 M potassium dihydrogen phosphate, 0.72 M dipotassium hydrogen phosphate) and kanamycin (50 μg mL^{-1}). After the pre culture reached an OD_{600} of 3 it was used to inoculate the fermenter medium supplemented with kanamycin (50 μg mL^{-1}).

The fermentation was done at 28 °C under control of dissolved oxygen (≥30%). The pH value of 7.0 was adjusted automatically by addition of phosphoric acid (10% (v/v)) and ammonia (25%). After 14 h, the cultures were induced with IPTG (1 mM). The feed (600 g L^{-1} glucose, 180 g L^{-1} yeast extract, 43.2 g L^{-1} ammonium chloride) was added with 1.4 mL/min within 12 h. After 26 h in total, the cells were harvested at 15,970 × *g* for 20 min. The cell pellets (NIT*Af* 247 g, pHNIT 162 g, 9/H6 317 g) were stored at 4 °C.

For HIS Tag affinity chromatographic purification, 20 g of cell pellet were resuspended in sodium phosphate buffer (20 mM, pH 7.5, 5 mL g^{-1} cell pellet) containing sodium chloride (500 mM). The cells were disrupted by ultrasonication. The cell debris was removed by ultracentrifugation at 1,64,685 × *g* for 45 min at 4 °C. The supernatant (crude lysate) was applied to a 60 mL Ni Sepharose 6 Fast Flow column (GE Healthcare, Great Britain) after filtration through a 45 μm syringe filter. The tagged enzymes were obtained by a one step purification using the buffers recommended in the manual, supplied with TCEP (tris(2-carboxyethyl) phosphine hydrochloride, 1 mM) as reducing agent. After purification, the enzyme solution was desalted against potassium phosphate buffer (10 mM, pH 7.5) using HiTrap Desalting columns (GE Healthcare, Great Britain). TCEP (1 mM) was added to the collected protein fractions which were then concentrated 8-fold by Vivaspin 20 tubes (10,000 MWCO, Sartorius). Finally, the enzyme solutions (approximately 15 mL) were lyophilized in 100 mL round bottom flasks using a Christ Alpha 1-4 LSC (Osterode, Germany) for 12 h at 0.570 mbar and −60 °C. The lyophilized enzyme preparations were stored at −20 °C.

2.4. Conversion of **1**

For determination of the specific activity a reaction mixture of potassium phosphate buffer (2.64 mL, 50 mM pH 7.5) and **1** (60 μL, 500 mM in 100% methanol, ε_{220} 0.3 mM^{-1}) was prepared. The reaction was started by adding 300 μL of 10 mg mL^{-1} lyophilized purified enzyme (dissolved in doubly distilled H_2O). Samples (100 μL) were taken for 10 min every min and added to HCl (10 μL, 2 M) to stop the reaction. After adding acetonitrile (50 μL), the samples were centrifuged for 10 min at 11,514 × *g*. The supernatant (100 μL) was transferred to a microtiterplate and analyzed by HPLC/MS with the following conditions: mobile phase: 60% acetonitrile, 40% formic acid (0.1% (v/v) in water), flow 1 mL min^{-1}, 40 °C. As stationary