Enzyme and Microbial Technology 61–62 (2014) 7–12

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

An alcohol oxidase of *Phanerochaete chrysosporium* with a distinct glycerol oxidase activity

Diana Linke[a,*], Nicole Lehnert[a], Manfred Nimtz[b], Ralf G. Berger[a]

[a] Institut für Lebensmittelchemie, Leibniz Universität Hannover, Callinstraße 5, D-30167, Hannover, Germany
[b] Helmholtz Zentrum für Infektionsforschung, Inhoffenstrasse 7, D-38124 Braunschweig, Germany

ARTICLE INFO

Article history:
Received 1 October 2013
Received in revised form 14 February 2014
Accepted 2 April 2014
Available online 23 April 2014

Keywords:
Alcohol oxidase
Glycerol
Phanerochaete chrysosporium

ABSTRACT

An intracellular alcohol oxidase (AOX) was isolated from the white-rot basidiomycete *Phanerochaete chrysosporium* (Pch), grown on L-lactate induction medium, and purified to electrophoretic homogeneity. The dimeric protein consisted of two identical 75 kDa subunits. The open reading frame of 1,956 bp resulted in a monomer consisting of 651 amino acids. The enzyme showed a p*I* at 5.4, a pH optimum of 9, a temperature optimum at 50 °C, possessed putative conserved domains of the GMC superfamily, a FAD binding domain, and showed up to 86% homology to alcohol oxidase sequences of *Gloeophyllum trabeum* and *Coprinopsis cinerea*. As was shown for the first time for an AOX from a basidiomycete, not only methanol, but also lower primary alcohols and glycerol were accepted as substrates. An assay based on aldehyde dehydrogenase confirmed D-glyceraldehyde as the product of the reaction. A bioprocess based on this enzyme could alleviate the problems associated with the huge side-stream of glycerol occurring during the manufacture of biodiesel, yielding the green oxidant hydrogen peroxide.

1. Introduction

Glycerol accumulates as a by-product of the production of biodiesel and soap during the transesterification or saponification of triacylglycerols. The rapidly increasing volumes stimulated attempts to biologically or chemically convert glycerol to propanediols, hydrogen, dihydroxyacetone, succinic acid or macromolecules (http://www.york.ac.uk/res/sustoil/). Glycerol would also present an abundant substrate for production of hydrogen peroxide, an environmentally favourable and potent oxidant, using a glycerol oxidase. The second product of the reaction, glyceraldehyde, is a multifunctional chiral building block and precursor of mono- and diterpenes in biosystems operating the non-mevalonate pathway.

Wood degrading white-rot basidiomycetes, such as *Phanerochaete chrysosporium*, form a wide array of redox enzymes depending on or producing H_2O_2 [1–6]. This set of enzymes includes alcohol oxidases (AOX), which catalyze the reaction of alcohols to the corresponding aldehyde and hydrogen peroxide. Peroxidases in turn, representing another part of the interlinked enzyme network, consume hydrogen peroxide as a co-substrate to attack lignin. Among the few oxidases of industrial importance is glucose oxidase, which is not only useful to produce hydrogen peroxide, but also to remove glucose or oxygen, thus acting as an inhibitor of non-enzymatic browning and as an antioxidant in food processing [7].

A number of AOX are known from basidiomycetes, for example from *Pleurotaceae* [3,4,6] such as *Pleurotus sajor-caju* [1] and *Pleurotus eryngii* [8], but also from *Bjerkandera adusta* [5], *Gloeophyllum trabeum* [9], *P. chrysosporium* [2,10,11], and from ascomycetes, such as *Penicillium purpurescens* [12]. All of these AOX oxidized common alcohols, such as methanol, ethanol, and 1-propanol, and often also lignin-related aryl-alcohols, such as veratryl-alcohol, but no glycerol.

A first glycerol oxidase was isolated from the ascomycete *Aspergillus japonicus* and thoroughly investigated [13–15]. Glycerol oxidases from *Penicillium* sp. [16] and from *Botrytis allii* [17] were described. Glycerol oxidase activities were also supposed to exist in strains of *Aspergillus*, *Neurospora* and *Penicillium* when grown on glycerol as the sole carbon source [14]. No glycerol oxidases or AOX with a side activity for glycerol are known from basidiomycetes. This motivated the search for a basidiomycetous glycerol oxidase and resulted in the characterization of such an enzyme in the present work.

* Corresponding author. Tel.: +49 511 7624583; fax: +49 511 7624547.
E-mail address: Diana.linke@lci.uni-hannover.de (D. Linke).

http://dx.doi.org/10.1016/j.enzmictec.2014.04.001
0141-0229/

2. Materials and methods

2.1. Chemicals

The chemicals and consumables were purchased in the required purity from Sigma (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Hannover, Germany), Fluka (Buchs, Switzerland), and Roth (Karlsruhe, Germany).

2.2. Cultivation of P. chrysosporium

Before use all media and equipment were autoclaved. Stock cultures of *P. chrysosporium* (DSMZ 1547) were cultivated in petri dishes on standard agar plates (30.0 g L^{-1} glucose monohydrate; 4.5 g L^{-1} L-asparagine monohydrate; 1.5 g L^{-1} KH_2PO_4; 0.5 g L^{-1} $MgSO_4$; 3.0 g L^{-1} yeast extract; 15.0 g L^{-1} agar agar; 1.0 mL L^{-1} trace element solution containing 0.005 g L^{-1} $CuSO_4{\cdot}H_2O$, 0.08 g L^{-1} $FeCl_3 \times 6H_2O$, 0.09 g L^{-1} $ZnSO_4 \times 7H_2O$, 0.03 g L^{-1} $MnSO_4 \times H_2O$, and 0.4 g L^{-1} EDTA). The pH of the medium was adjusted to pH 6 with 1 M NaOH before sterilization.

A 1- cm^2 piece was cut out of the stock culture and transferred in 125 mL of SNL medium (same medium without agar agar) and homogenized using an Ultra-Turrax. After incubation at 24 °C and 150 rpm in an incubator for 6–10 days, 25 mL of pre-culture were transferred in 225 mL of lactate medium (10.0 g L^{-1} sodium L-lactate; 4.5 g L^{-1} L-asparagine monohydrate; 1.5 g L^{-1} KH_2PO_4; 0.5 g L^{-1} $MgSO_4$; 3.0 g L^{-1} yeast extract; 1.0 mL L^{-1} trace element solution) and incubated at 24 °C.

After 16 days of cultivation, the culture was filtrated and the biomass was washed with Tris–HCl buffer, 20 mM pH 7.0 and finally stored at −18 °C.

2.3. Enzyme purification from P. chrysosporium

10 g of biomass were homogenized with Tris–HCl 200 mM pH 7.0 using a Precellys 24 bead mill (peqlab, Erlangen, Germany). After centrifugation for 5 min at 16,000 × *g* the supernatant was used for further purification. The solution was applied to a Superdex 75 column and elution was performed with Tris–HCl 200 mM pH 7.0 at a flow rate of 0.5 mL min^{-1}. Fractions of 0.5 mL were collected and analyzed for activity.

2.4. Activity assay with aminoantipyrine

The assay of Uwajima and Tereda [18] was used for the determination of H_2O_2. Using 4-aminoantipyrine, phenol and horseradish peroxidase, H_2O_2 reacts to a pink quinoid product that is measured at 500 nm The assay was carried out with Tris–HCl 200 mM, pH 7.0. Blinds were carried out (1) without enzyme sample, (2) without glycerol and (3) by inactivating the enzyme sample for 10 min at 95 °C. Formation of product was followed using a microplate reader. One unit of enzyme activity was calculated as the amount of enzyme required to produce 1 μM H_2O_2, respectively, at 30 °C per minute.

2.5. Temperature and pH optima

For determination of the pH-optimum an assay using ABTS 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt was performed at pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using Tris base and piperazine buffer, respectively. The assay was performed at 30 °C in 96 well plates for a high sample throughput. Following approach was used for the ABTS assay: 150 μL buffer 400 mM, 75 μL sample, 30 μL glycerol 100 mM, 30 μL HRP 100 U mL^{-1} and 15 μL ABTS were mixed. The increase of extinction was observed at 420 nm for 20 min. Each sample was measured in triplicates. To determine the temperature optimum of the alcohol oxidase the ABTS assay was exercised at 30, 37, 42 and 50 °C.

2.6. ESI-Tandem MS analysis of tryptic peptides

The enzyme band was excised from SDS polyacrylamide gels, dried, and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. An Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanospray ion source and gold-coated capillaries was used for electrospray ionization (ESI) MS of peptides. For collision-induced dissociation experiments, multiple charged parent ions of interest were selectively retained in the linear ion trap, then transferred to the external collision cell and the obtained fragment ions detected in the Orbitrap analyzer. MS^3 spectra of selected fragments were recorded to obtain complete sequence information if necessary. From these spectra, the amino acid sequences were manually deduced and used for cross-species protein identification in public protein primary sequence databases.

2.7. Native gradient and denaturing SDS-PAGE

For Native gradient PAGE self-casted 1 mm thick polyacrylamide single gradient gels with a concentration of 3–12% were used for the gel electrophoresis. 20 μL of enzyme samples were mixed with 10 μL of the native loading buffer (0.05 M Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue) and were separated at 4 °C and 10 mA per gel in a Laemmli buffer system. For estimation of the size the All Blue Precision Plus pre-stained protein marker (Biorad, Germany) was used. After the electrophoresis an activity overlay was prepared by mixing 7.5 mL Tris–HCl 400 mM, pH 7.0, 1.5 mL glycerol (50% (v/v)), 1.5 mL aminoantipyrine 4 mM, 1.5 mL phenol 70 mM, 1.5 mL HRP 15 U mL^{-1} and 225 mg agarose. The hand-hot agarose gel matrix was casted onto the Native gel and enzyme activity appeared after incubation at 37 °C as pink bands.

For denaturing SDS-PAGE samples were prepared by mixing 2:1 (v/v) with loading buffer (0.1 M Tris–HCl (pH 6.8), 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and boiling for 15 min. After electrophoresis at 20 mA per gel, the gels were stained with Coomassie Brilliant Blue.

2.8. Isoelectric focusing

IEF agarose gel electrophoresis was performed on a Multiphor II system (Pharmacia LKB, Sweden). Gels of Biozym® pH 3–10 and a protein standard of Servalyt precotes® (pI 3–10) were used for the estimation of the isoelectric point. The gels are suitable for proteins larger than 200 kDa. 5 μL of sample and standard, respectively, were applied on the gel. Electrophoresis was performed at 3 W, 1500 V, 12.5 mA for 5 h. Afterwards the gel was activity stained with an overlay of aminoantipyrine and phenol. Protein bands were stained with Coomassie R.

2.9. Protein content

The protein content was estimated by measuring the absorbance at 750 nm using Lowry method [19].

2.10. Stereochemistry of the glyceraldehyde product

To verify glyceraldehyde as a product of the reaction, a stereoselective aldehyde dehydrogenase (ALDH) from baker's yeast (Sigma Aldrich, St. Louis, MO, USA) was used in a spectrophotometric assay. ALDH oxidizes D-glyceraldehyde to glyceric acid in the presence of NAD^+. The reaction mixture consisted of 30 μL NAD^+ (3.5 mM), 30 μL glycerol (200 mM), 1.5 U ALDH, and 50 μL glycerol oxidase sample ad 300 μL Tris–HCl buffer (100 mM, pH 9). Several blanks (sample + D/L-glyceraldehyde, ALDH + glycerol, sample + glycerol without NAD^+, ALDH + D/L-glyceraldehyde without NAD^+) and a positive control (D/L-glyceraldehyde + ALDH) were prepared, and the rates of reduction of NAD^+ were monitored at 340 nm and 30 °C using a microplate reader.

2.11. First-strand cDNA-synthesis

RNA was prepared from 500 mg mycelium stored in RNALater® (Invitrogen, Darmstadt, Germany) using the NucleoSpin®RNA Plant Kit (Macherey-Nagel, Düren, Germany).

Five micrograms total RNA were mixed with 25 pmol 3′ Primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG 30*T VN-3′) and filled up to 10 μL with H_2O. The mixture was incubated at 70 °C for 10 min and then chilled on ice for 2 min. 4 μL 5× reaction buffer, 2 μL dNTP mix (10 mM each), 1 μL RiboLock™, 1 μL $MnCl_2$ (160 mM) and 200 U RevertAid™ H Minus M-MuLV Reverse Transcriptase were added, mixed and incubated at 42 °C for 60 min and at 50 °C for 10 min. Termination was carried out by heating at 70 °C for 5 min.

Enzymes and reagents were purchased from Fermentas, St. Leon-Rot, Germany; oligonucleotides were synthesized by Eurofins MWG Operon, Ebersberg, Germany.

2.12. Sequence fishing

Primers were deduced from the published genome of *P. chrysosporium*: start_*Pch* 5′-ATGGGTCACCCCGAGGAG-3′ and stop_*Pch* 5′-TTACCGGACCTGCTGGGTAG-3′. PCRs were performed by mixing 2 μL High Fidelity buffer 10×, 0.4 μL dNTP mix (2.5 mM each), 25 pmol forward primer, 25 pmol reverse primer, five U High Fidelity Enzyme Mix (Fermentas), 1 μL first-strand cDNA and ddH_2O to 20 μL at an annealing temperature of 56 °C and an elongation step of 2.5 min at 72 °C (30 cycles).

PCRs were analyzed by agarose gel electrophoresis [1% agarose (Serva, Heidelberg, Germany) cooked in TAE-buffer (40 mM Tris–HCl, 20 mM acetic acid, 1 mM EDTA pH 8)]. For detection of DNA, 0.05‰ SYBRSafe™ (Invitrogen) was added to the solution after it cooled down to about 50 °C. DNA extraction from agarose gels was carried out with the NucleoSpin Extract II Kit (Macherey-Nagel).

DNA fragments were ligated into the pGEM®T-Vector (Promega). 7 μL of insert, 1 μL of vector, 5 U T4 DNA Ligase and 1 μL of T4 DNA Ligase buffer 10x were mixed and incubated for 16 h at 14 °C.

For transformation 5 μL ligation reaction were added to 50 μL chemically competent *E. coli* TOP10 (Invitrogen), incubated on ice for 20 min, heat shocked at 37 °C for 5 min and transferred on ice immediately. 250 μL of warm SOC medium (Invitrogen) was added. The cells were incubated at 37 °C for 30–60 min at 225 rpm. 50 μL of the cells were spread out on LB-agar containing 50 μg mL^{-1} ampicillin and 20 μg mL^{-1} X-Gal. The plates were incubated for 16 h at 37 °C.

Selection of positive clones was performed by colony PCR. The reaction mixture was composed as stated above but primers M13 uni (-21) (5′-TGTAAAACGACGGCCAGT-3′) and M13 rev (-29) (5′-CAGGAAACAGCTATGACC-3′) were used. Template was added by resuspending white colony material in the reaction mixture which was then incubated at 95 °C for 10 min, followed by 35 cycles