Bioresource Technology 170 (2014) 445–453

Contents lists available at ScienceDirect

Bioresource Technology

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

Selective oxidation of glycerol to 1,3-dihydroxyacetone by covalently immobilized glycerol dehydrogenases with higher stability and lower product inhibition

Javier Rocha-Martin [a], Andreína Acosta [a], Jose Berenguer [b], Jose M. Guisan [a], Fernando Lopez-Gallego [c,d,*]

[a] Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica-CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain
[b] Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Campus UAM, Cantoblanco, 28049 Madrid, Spain
[c] Biofunctional Nanomaterials Unit, CIC BiomaGUNE, Paseo Miramon 182, 20009 San Sebastian, Spain
[d] IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

HIGHLIGHTS

- Covalent immobilization stabilizes glycerol dehydrogenase from different sources.
- Orientation through the most acid area of GlyDH minimizes its product inhibition.
- Covalent post-immobilization cross-linking rather improves enzyme properties.

ARTICLE INFO

Article history:
Received 2 June 2014
Received in revised form 30 July 2014
Accepted 31 July 2014
Available online 9 August 2014

Keywords:
Glycerol dehydrogenase
Enzyme immobilization
Dihydroxyacetone and protein stabilization

ABSTRACT

Glycerol dehydrogenase (GlyDH) catalyzes the regioselective oxidation of glycerol to yield 1,3-dihydroxyacetone (DHA); an important building block in chemical industry. Three recombinant GlyDHs from *Geobacillus stearothermophilus*, from *Citrobacter braakii* and from *Cellulomonas* sp. were stabilized by covalent immobilization. The highest activity recoveries (40–50%) of the insoluble preparations were obtained by immobilizing these enzymes in presence of polyethylene glycol (PEG). Noteworthy, these immobilized preparations were more stable and less inhibited by DHA than their soluble counterparts. In particular, GlyDH from *G. stearothermophilus* immobilized on agarose activated with both amine and glyoxyl groups and crosslinked with dextran aldehyde was 3.7-fold less inhibited by DHA than its soluble form and retained 100% of its initial activity after 18 h of incubation at 65 °C and pH 7. This is one of the few examples where the same immobilization protocol has minimized enzyme product inhibition and maximized thermal stability.

1. Introduction

Glycerol is an important industrial chemical with many applications in cosmetics, paint, food, tobacco, pharmaceutical, leather and textile industries (Pagliaro et al., 2007; Wang et al., 2001). Nevertheless, due to the large excess of glycerol formed as a by-product during the production of biodiesel, considerable efforts are being devoted to convert glycerol into other value-added chemicals such as 1,3-propanediol, 2,3-butanediol, glyceric acid, or 1,3-dihydroxyacetone (DHA) (Enders et al., 2005; Pagliaro et al., 2007). DHA, with a market price over 100-fold that of glycerol, is a valuable chemical with a wide range of applications in cosmetics (Wang et al., 2001; Zheng and Zhang, 2011), food and pharmacy industries (Suga et al., 2002) and also is a very important C_3 building block in organic synthesis (Enders et al., 2005). Moreover, lowering the cost of DHA may eventually open the avenue for the synthesis of new biodegradable polymers (Weiser et al., 2011).

Microbial biotransformation for DHA production has been revealed more cost-effective and more environmentally friendly than the chemical synthesis (Hekmat et al., 2007). However, the microbial process presents some productivity issues that limit

Abbreviations: GlyDH, glycerol dehydrogenase; PEG, polyethylene glycol; DHA, dihydroxyacetone; GlyDH-C, glycerol dehydrogenase from *Cellulomonas* sp.; GlyDH-Cb, glycerol dehydrogenase from *Citrobacter braakii*; NOX, NADH oxidase from *Thermus thermophilus*; GlyDH-Gs, glycerol dehydrogenase from *Geobacillus stearothermophilus*; IC_{50}, DHA concentration which inhibits 50% of GlyDH activity.

* Corresponding author at: Biofunctional Nanomaterials Unit, CIC BiomaGUNE, Paseo Miramon 182, 20009 San Sebastian, Spain. Tel.: +34 943005300x309; fax: +34 943005301 (F. López-Gallego).

E-mail addresses: jmguisan@icp.csic.es (J.M. Guisan), flopez.ikerbasque@cicbiomagune.es (F. Lopez-Gallego).

http://dx.doi.org/10.1016/j.biortech.2014.07.116
0960-8524/

broader applications. These drawbacks are related to long fermentation times, high energy consumption for sterilization, and limitations due to high oxygen requirement, as well as severe inhibitory effects on cell growth from high concentrations of both substrate and product (Hekmat et al., 2007). Alternatively, glycerol oxidation catalyzed by isolated enzymes can overcome some of these drawbacks and provides the DHA production process with unique selectivity and high efficiency. In this regard, glycerol dehydrogenase (GlyDH) (EC 1.1.1.6) is a NAD^+-dependent oxidoreductase that converts glycerol into DHA. Nevertheless, the high production cost of isolated enzymes, their low stability and their requirements for expensive redox cofactors have limited their industrial exploitation. In addition, strong inhibition of wild type GlyDH by DHA dramatically hampers high titers of this product in batch reactors without *in situ* product extraction (Zhang et al., 2011; Zheng and Zhang, 2011). Therefore, the underlying product inhibition and low stability of GlyDH are the most critical drawbacks for GlyDH industrial application. These limitations thus encourage scientists to seek new tools to diminish enzyme product inhibition and increase enzyme stability. In this context, immobilization of GlyDH on solid carriers is a plausible solution to improve enzyme properties (Mateo et al., 2007). However, encountering the optimal immobilization protocol is rather challenging since the vast majority of GlyDHs found in nature are multimers. This means that both tertiary and quaternary structures must be stabilized.

For the last 50 years many immobilization protocols have been successfully applied to enzymes. These solid biocatalysts can be easily re-used for several cycles increasing the cost-efficiency of the biotransformation (Alvaro et al., 1990). Immobilization techniques may also promote enzyme stabilizations that would increase enzyme life-time and therefore their potential as industrial catalysts (Mateo et al., 2006). Additionally, it has been demonstrated how some immobilization protocol promoted slight changes in the active center conformation reducing either product or substrate inhibition of the resulting immobilized enzymes (Pessela et al., 2003).

In this study, three recombinant and multimeric GlyDHs from different microbial sources (*Citrobacter braakii*, *Geobacillus stearothermophilus* and *Cellulomonas* sp.) were covalently immobilized on agarose-type carriers. Agarose carriers have shown highly compatible with proteins due to their hydrophilic and inert nature, their versatility to be activated with different reactive groups and their suitable porosity to achieve high volumetric activities of the resulting insoluble biocatalysts. We have screened different immobilization chemistries and conditions to select the most stable and less inhibited immobilized biocatalyst for the oxidation of glycerol to DHA. This work is one of the few examples where immobilization minimizes enzyme product inhibition at the same time that maximizes the enzyme thermal stability.

2. Methods

2.1. Materials

Nicotinamide adenine dinucleotide derivatives (NAD^+ and NADH) were purchased from GERBU Biotechnik GmbH (Wieblingen, Germany). Glycerol dehydrogenase from *Cellulomonas* sp. (GlyDH-C), glycidol, glycerol, triethylamine (TEA), polyethylene glycol (PEG), 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), sodium borohydride, sodium periodate were supplied by Sigma–Aldrich Co (St. Louis, IL). 1,3-dihydroxyacetone was supplied by Acros Organics (Geel, Belgium). Cyanogen-bromide-activated Sepharose 4B (Ag-CB) and low molecular weight electrophoresis markers were purchased from GE Healthcare (Uppsala, Sweden). Cross-linked agarose beads (4%) were from Agarose Beads Technology (Madrid, Spain). Glycerol dehydrogenase (GlyDH-Cb) from *C. braakii* was produced and purified as published elsewhere (Rocha-Martín et al., 2012a). The Coomassie (Bradford) protein assay kit was purchased from Pierce (Rockford, Illinois, USA). All other used reagents were of analytical grade.

2.2. Preparation of the different agarose supports

Monoaminoethyl-N-aminoethyl agarose (Ag-MANAE) lowly activated used for the GlyDH-Gs purification was prepared as described elsewhere (Pessela et al., 2006). Agarose beads activated with glyoxyl groups (Ag-G) and agarose beads activated with both amine and glyoxyl groups (Ag-AG) were prepared as previously described by (Guisán, 1988) and (Mateo et al., 2010) respectively.

2.3. Cloning of a Glycerol dehydrogenase from G. stearothermophilus (GlyDH-Gs)

2.3.1. 1. Bacterial strains and growth conditions

Laboratory stocks of *Escherichia coli* DH10B and BL21 (DE3) strains were used to produce chemical competent bacteria that were used for cloning and expression purposes, respectively. *E. coli* strains were aerobically cultured in Luria–Bertani (LB) medium at 37 °C.

2.3.2. Cloning and overexpression of dhaD gene

DNA isolation, plasmid purification, restriction analysis, plasmid construction and DNA sequencing were carried out by standard methods (Sambrook et al., 1989). The gene encoding a glycerol dehydrogenase from *G. stearothermophilus* (*glydh-gs*) was PCR-amplified from *G. stearothermophilus* genomic DNA purchased from ATCC (Americal Type Culture Collection). We used primers GlyDH-5′-TAACATATGGCGGCAGAAAGAGTA-3′ as forward and GlyDH-5′-TGCGAATTCAAAACGTTATTTGCG-3′ as reverse that contain restriction sites for the enzymes NdeI and EcoRI (underlined). The purified PCR product was digested with these restriction enzymes and further cloned into the pET28b vector cut with the same restriction enzymes. We named the resulting plasmid bearing *glydh-gs* gene as pET28b-*glydh-gs*.

2.3.3. Production of the recombinant GlyDH-Gs in E. coli

E. coli BL21 (DE3) cells were transformed with the recombinant plasmid pET28b-GlyDH-Gs. Cells containing such plasmid were grown at 37 °C in LB medium supplemented with kanamycin (50 μg ml^{-1}) and induced by adding 1 mM Iso-propyl-1-Thio-β-D-galactopyranoside (IPTG) when the culture reached an OD_{600} = 0.5. After 4 h at 37 °C the cells were collected by centrifugation (10,000×*g*, 10 min).

2.3.4. Purification of the GlyDH-Gs

For protein purification, harvested cells were resuspended in purification buffer (5 mM sodium phosphate at pH 7.0). The cells were lysed by sonication, and the extract was centrifuged at 12000 rpm to remove the cell debris. The resulting soluble crude extract was subjected to anionic exchanger chromatography in Ag-MANAE equilibrated with the purification buffer. Following to the protein binding, the column was washed three times with purification buffer prior to the elution with 300 mM NaCl.

2.4. Enzymatic activity assays

The activities of the different GlyDH preparations was analyzed spectrophotometrically recording the increment of absorbance at 340 nm (ε_{NADH} = 6.22 mM^{-1} cm^{-1}) promoted by the formation of NADH during the oxidation of glycerol. A sample of enzymatic preparation (10–200 μL) was added to a cell with 2 mL of