Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2016

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Spectrophotometric assay for sensitive detection of glycerol dehydratase activity using aldehyde dehydrogenase

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Received 9 September 2016; accepted 3 December 2016
Available online xxx

Glycerol dehydratase (GDHt) is a pivotal enzyme for fermentative utilization of glycerol by catalyzing radical-mediated conversion of glycerol into 3-hydroxypropionaldehyde (3-HPA). Precise and sensitive monitoring of cellular GDHt activity during the fermentation process is a prerequisite for reliable metabolic analysis to afford efficient cellular engineering and process optimization. Here we report a new spectrophotometric assay for the sensitive measurement of the GDHt activity with a sub-nanomolar limit of detection (LOD). The assay method employs aldehyde dehydrogenase (ALDH) as a reporter enzyme, so the readout of the GDHt activity is recorded at 340 nm as an increase in UV absorbance which results from NADH generation accompanied by oxidation of 3-HPA to 3-hydroxypropionic acid (3-HP). The GDHt assay was performed under the reaction conditions where the ALDH activity overwhelms the GDHt activity (i.e., 50-fold higher activity of ALDH relative to GDHt activity), affording sensitive detection of GDHt with 360 pM LOD. The ALDH-coupled assay was used to determine kinetic parameters of GDHt for glycerol, leading to K_M = 0.73 ± 0.09 mM and k_{cat} = 400 ± 20 s^{-1} which are in reasonable agreements with the previous reports. Our assay method allowed measurement of even a 10^4-fold decrease in the cellular GDHt activity during fermentative production of 3-HP, which demonstrates the detection sensitivity much higher than the previous methods.

[**Key words:** Glycerol dehydratase; Glycerol; Aldehyde dehydrogenase; Biocatalysis; Enzyme assay; Bioprocess monitoring]

Manufacturing of transportation fuels and commodity chemicals from renewable feedstocks has received increasing attention owing to shrinking oil reserves available for conventional petroleum industry (1). The past decade has witnessed unprecedented growth in biodiesel production, leading to a large surplus of glycerol as a by-product (2,3). The cheap crude glycerol derived from the biodiesel industry has been recognized as a sustainable feedstock for fermentative production of 1,3-propanediol (1,3-PDO) and 3-hydroxypropionic acid (3-HP) which are used as a monomer for a wide range of polymers and a platform chemical for production of a number of commodity chemicals, respectively (4,5).

Glycerol dehydratase (GDHt, EC 4.2.1.30) is a key enzyme for the bioconversion of glycerol into the value-added chemicals and thereby has recently attracted a great deal of attention as an essential component for metabolic engineering (4,5). GDHt carries out a radical-mediated turnover of glycerol into 3-hydroxypropionaldehyde (3-HPA) using adenosylcobalamin (i.e., coenzyme B_{12}) as a cofactor (6). 3-HPA is further processed to either 1,3-PDO or 3-HP, depending on the type of the oxidoreductase acting on the aldehyde group of 3-HPA (Fig. 1). Radical formation in the adenosylcobalamin moiety by a homolytic cleavage of the Co–C bond initiates the radical catalysis, leading to dehydration of the bound substrate (6). It is known that GDHt undergoes mechanism-based inactivation during the catalysis and the resulting damaged cofactor is unable to mediate the radical reaction (7). Owing to the pivotal role of GDHt in the glycerol conversion, reliable metabolic engineering strategies to cope with the undesirable inactivation and to optimize the enzyme expression level have been considered as one of the crucial factors to the successful process design. To this end, a comprehensive metabolic analysis should be guided by precise and sensitive monitoring of the change in the GDHt activity throughout the fermentation process.

To date, two assay methods have been widely employed to quantify the GDHt activity. One method, developed by Toraya et al., is an end-point assay where 3-HPA produced from the GDHt reaction is modified with 3-methyl-2-benzothiazolinone hydrazone (MBTH) under acidic conditions and turns into a colored compound detectable at 305 nm with a molar extinction coefficient of 1.33×10^4 M^{-1} cm^{-1} (8). Despite the high detection sensitivity for 3-HPA, the MBTH method is not compatible with real-time monitoring of the 3-HPA generation and thereby is less suitable for kinetic studies that require accurate initial rate measurements. In contrast, the other method, developed by Yakusheva et al. (9), employs a coupled enzyme assay, using alcohol dehydrogenase (ADH), which allows real-time monitoring of the GDHt activity. In this method, 3-HPA is continuously converted to 1,3-PDO by ADH and the ensuing conversion of NADH to NAD^+ is recorded as a decrease in the UV absorbance at 340 nm (Abs_{340}) with a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. Considering the

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.12.002

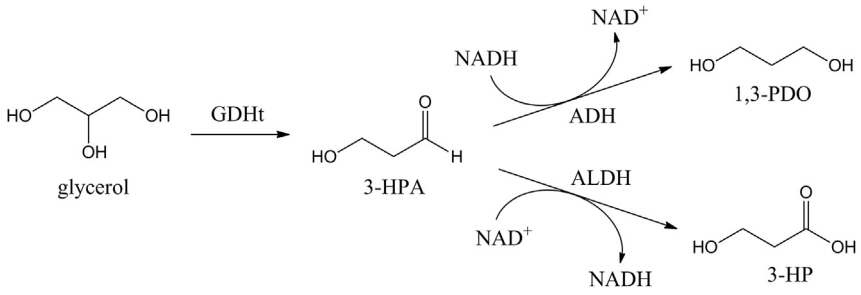

FIG. 1. Conversion of glycerol to either 1,3-PDO or 3-HP via 3-HPA. ADH and ALDH stand for alcohol dehydrogenase and aldehyde dehydrogenase, respectively.

Michaelis constant (i.e., K_M) of commercially available yeast ADH for NADH around 0.1 mM (10), a standard assay protocol to saturate ADH with the cofactor mandates use of at least 0.2 mM NADH which corresponds to $Abs_{340} > 1.2$ (11). Therefore, the ADH method does not allow use of a high dose of crude cell extract in the assay mixture because of signal saturation that could result from background UV absorption of the crude cell extract. This signal saturation problem significantly deteriorates detection limit of the ADH method for quantitation of a very low level of GDHt in the crude cell extract.

In this report we aimed at developing a convenient continuous assay for sensitive detection of the GDHt level without being interfered by the background UV absorbance in the crude cell extract, so the assay method is available for precise monitoring of a dynamic change in the GDHt expression level during fermentation process. We reasoned that the aforementioned drawback of the ADH-coupled assay method could be circumvented by using aldehyde dehydrogenase (ALDH) because ALDH-catalyzed oxidation of 3-HPA to 3-HP requires NAD^+. The use of the oxidized form of the cofactor rendered the initial UV absorbance in the assay mixture low, allowing use of a high concentration of crude cell extract without the signal saturation.

MATERIALS AND METHODS

Expression plasmids and bacterial cells Expression plasmids used in this study are listed in Table 1. *Escherichia coli* BL21(DE3) cells transformed with a pCBG2 plasmid were used for preparation of crude cell extract of which the GDHt activity was assayed. The low-copy-number pCBG2 plasmid was constructed previously and harbors the genes encoding GDHt (i.e., *dhaB*) and reactivase (i.e., *gdrAB*) cloned from *Klebsiella pneumoniae* DSM 2026 (12).

For preparation of purified GDHt, the *dhaB* gene was cloned in a high-copy-number plasmid. The *dhaB* gene was amplified from the pCBG2 plasmid and cloned into a pET28a(+) vector (Novagen, Madison, WI, USA). PCR primers used for the gene amplification were forward 5′-AGAAGGAGATATACCATGAAAAGATCAAAACGA-3′ and reverse 5′-GGTGGTGGTG GTGCTCGAGGCTTCCTTTACGCAGCTT-3′. The PCR product was ligated with a *NcoI*/*XhoI* cut of pET28a(+) using an Infusion HD cloning kit (Clontech, Mountain View, CA, USA). The resulting cloning product (i.e., pGDHt) was confirmed by DNA sequencing. *E. coli* BL21(DE3) cells transformed with the pGDHt plasmid was used for preparation of the purified GDHt.

Preparation of purified ALDH used for activity assay of GDHt was carried out with *E. coli* BL21(DE3) cells transformed with a pQAD2 plasmid. The pQAD2 plasmid harbors *aldH* cloned previously from *E. coli* K-12 MG1655 (13).

TABLE 1. Expression plasmids used in this study.

Plasmid	Description	Source
pCBG2	*dhaB* and *gdrAB* in pCDFDuet-1 vector; T7 promoter; *lacI*; Sm^r	12
pGDHt	*dhaB* in pET28a(+) vector; T7 promoter; *lacI*; Kan^r	This study
pQAD2	*aldH* in pQE-80L; T5 promoter; *lacI*; Amp^r	13
pRKS1	*KGSADH* in pRSFDuet-1; T7 promoter; *lacI*; Kan^r	12

E. coli BL21(DE3) cells co-transformed with the pCBG2 and pRKS1 plasmids were used for fermentative conversion of glycerol to 3-HP as reported previously (12). The pRKS1 plasmid was constructed previously and harbors α-ketoglutaric semialdehyde dehydrogenase (KGSADH) (12).

Preparation of crude cell extract and purified enzyme *E. coli* BL21(DE3) cells carrying an expression vector were cultivated in LB medium (typically 1 L) supplemented with appropriate antibiotics. Protein expression was induced by IPTG (final concentration = 1 mM) at 0.5 OD_{600} and the cells were allowed to grow for 10 h. The culture broth was centrifuged (10,000 ×*g*, 20 min, 4°C) and the resulting cell pellet was subjected to ultrasonic disruption or lysozyme treatment. For the ultrasonic disruption, cell pellet was resuspended in 20 mL sonication buffer (50 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 50 mM Tris–HCl and pH 7). Cells were lysed by ultrasonic disruption and then the supernatant solution obtained after centrifugation (17,000 ×*g*, 30 min, 4°C) was employed as cell-free extract for enzyme assays. For the lysozyme lysis, cell pellet were resuspended in 20 mL lysozyme buffer (10 mM Tris–HCl, pH 8, 0.1 M NaCl and 0.5 mg/mL chicken egg-white lysozyme). The cell lysis mixture was incubated in a shaking incubator at 37°C for 1 h and then crude cell extract was obtained after centrifugation (17,000 ×*g*, 30 min, 4°C). Total protein concentration in the crude cell extract was determined by the Bradford assay.

Purification of GDHt and ALDH was performed on ÄKTAprime plus (GE Healthcare, Piscataway, NJ, USA) with the crude cell extract obtained by ultrasonic cell disruption. His_6-tagged proteins were purified as described previously with minor modifications (14). Briefly, the His_6-tagged proteins were isolated from the crude cell extract using a HisTrap HP column (GE Healthcare). Removal of imidazole was carried out by a HiTrap desalting column (GE Healthcare) using an elution buffer (0.15 M sodium chloride, 50 mM sodium phosphate and pH 7). Molar concentrations of the purified enzyme solutions were determined from UV absorbance at 280 nm using molar extinction coefficients obtained by a protein extinction coefficient calculator (http://www.biomol.net/en/tools/proteinextinction.htm). Molar extinction coefficients used for the concentration determination were 118,330 and 246,680 M^{-1} cm^{-1} for GDHt and ALDH, respectively.

Enzyme assay Unless otherwise specified, enzyme assays were carried out at room temperature and pH 7 (50 mM potassium phosphate buffer) by measuring UV absorbance of NADH at 340 nm (extinction coefficient = 6.22×10^3 M^{-1} cm^{-1}) on a UV spectrophotometer (DU 650, Beckman Coulter Inc., Brea, CA, USA). One unit of ALDH was defined as the amount of enzyme required to reduce 1 μmole of NAD^+ to NADH in 1 min at 2 mM benzaldehyde and 2 mM NAD^+. One unit of GDHt was defined as the enzyme amount required to convert 1 μmole of glycerol to 3-HPA in 1 min at 40 mM glycerol and 1.5 μM coenzyme B_{12}. The enzyme activity of GDHt was measured in the presence of 2 mM NAD^+ and 5 U/mL purified ALDH, so the 3-HPA formed from glycerol was instantaneously converted to 3-HP by ALDH and the ensuing change in the UV absorbance at 340 nm was interpreted as the consumption of glycerol.

LOD for GDHt was calculated from linear regression of GDHt concentration against GDHt activity according to the guideline by International Conference on Harmonization: LOD = $3.3\sigma/S$ where σ is a standard deviation of y-intercept and *S* is a slope of the regression curve (15).

Kinetic measurement To determine kinetic parameters of GDHt for glycerol at room temperature, initial reaction rates were measured at varying concentrations of glycerol (0.2–10 mM), 1.5 μM coenzyme B_{12}, 2 mM NAD^+, 5 nM purified GDHt, 5 U/mL ALDH and 50 mM potassium phosphate (pH 7). The initial rate data were fitted to a Michaelis–Menten equation and the K_M and k_{cat} values were calculated from the slopes and y-intercepts of the double-reciprocal plots.

Monitoring of the GDHt activity during glycerol fermentation *E. coli* BL21(DE3) cells co-transformed with the plasmids pCBG2 and pRKS1 were cultivated in 50 mL M9 medium (4 g/L glucose, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.24 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 12.8 g/L $Na_2HPO_4 \cdot 7H_2O$ and 3 g/L KH_2PO_4) supplemented with streptomycin and kanamycin (both 50 μg/ml) as described before with minor modifications (12). The cells were grown aerobically in a 250 mL flask at 37°C and 250 rpm. When the cell OD_{600} reached 0.5, the culture broth was supplemented with IPTG, glycerol and coenzyme B_{12} (final concentrations = 0.1 mM, 0.1 M and 4 μM, respectively). Aliquots of the fermentation broth (1–15 mL) were taken at

Please cite this article in press as: Park, E.-S., et al., Spectrophotometric assay for sensitive detection of glycerol dehydratase activity using aldehyde dehydrogenase, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.12.002