Biotechnol Lett (2012) 34:1519–1523
DOI 10.1007/s10529-012-0933-4

ORIGINAL RESEARCH PAPER

Cloning, expression and characterization of *meso*-2,3-butanediol dehydrogenase from *Klebsiella pneumoniae*

Gen Lin Zhang · Cui Wei Wang · Chun Li

Received: 18 December 2011 / Accepted: 16 April 2012 / Published online: 1 May 2012

Abstract The *bud*C gene encoding the *meso*-2,3-BDH from *Klebsiella pneumoniae* XJ-Li was expressed in *E. coli* BL21 (DE3) pLys. Hypothetical amino acid sequence alignments revealed that the enzyme belongs to the short chain dehydrogenase/reductase family. After purification and refolding, the recombinant enzyme had activities of 218 U/mg for reduction of acetoin and 66 U/mg for oxidation of *meso*-2,3-butanediol. Highest activities were at pH 8.0 and 9.0 respectively. These are higher than other *meso*-2,3-butanediol dehydrogenases from *K. pneumoniae*. The low K_m value (0.65 mM) for acetoin indicated that the enzyme can easily reduce acetoin to *meso*-2,3-butanediol. There were no significant activities towards 2R,3R-2,3-butanediol, 1,4-butanediol and 2*S*,3*S*-2,3-butanediol, suggesting that the enzyme has a high stereospecificity for the *meso*-dihydric alcohol.

G. L. Zhang · C. W. Wang · C. Li (✉)
School of Life Science, Beijing Institute of Technology, Beijing 100081, China
e-mail: lichun@bit.edu.cn

G. L. Zhang
e-mail: zhgl_food@sina.com

C. W. Wang
e-mail: wangcuiwei19870115@163.com

G. L. Zhang
Key Laboratory for Green Processing of Chemical Engineering of Xinjiang Bingtuan, Shihezi University, Shihezi 832003, China

Keywords 2,3-Butanediol dehydrogenase · Enzymatic properties · Expression · *Klebsiella pneumoniae* · Substrate specificity

Introduction

2,3-Butanediol is a bulk chemical which can be produced through biotechnological route. It has been given much attention because of its potential application in butadiene and liquid fuel (Wardwell et al. 2001). Three stereoisomers of 2,3-butanediol (2*R*,3*R*-*meso*- and 2*S*,3*S*-forms) can be produced by different microorganisms, including *Klebsiella pneumoniae* (Ma et al. 2009), *Enterobacter aerogenes* (Converti et al. 2003), *Klebsiella oxytoca* (Ji et al. 2008), *Aeromonas hydrophila* (Van Houdt et al. 2007) and *Bacillus subtilis* (Nicholson 2008). Interestingly, these stereoisomers are synthesized by the homologous pathways involving pyruvate in a mixed acid fermentation process. α-Acetolactate, diacetyl, and acetoin are the main three intermediate compounds (Celińska and Grajek 2009). 2,3-Butanediol is formed by the reduction of acetoin with 2,3-butanediol dehydrogenase (BDH)/acetoin reductase.

Several 2,3-butanediol dehydrogenases vary in their stereospecificities among different strains. For example, *Saccharomyces cerevisiae* YAL060W and *K. pneumoniae* express *meso*-2,3-butanediol dehydrogenase (*meso*-2,3-BDH; Ui et al.1999; González et al. 2000), whereas *Brevibacterium saccharolyticum* produces 2*S*,3*S*-2,3-BDH (Ui et al. 2004). Previous

studies have also indicated that 2,3-BDH undergoes two kinds of enzyme activities (i.e., reduction and oxidation) in some cases. For example, the *meso*-2,3-BDH produced by *K. pneumoniae* is involved in the interconversion between acetoin and 2,3-butanediol (Ui et al.1999; Celińska and Grajek 2009).

Our previous study revealed that *meso*-2,3-butanediol is produced by *K. pneumoniae* XJ-Li (Zhang et al. 2011). To understand the characteristics of *meso*-2,3-BDH produced by *K. pneumoniae* XJ-Li and conduct a study on fermentation, the *meso*-2,3-BDH gene (*bud*C) was cloned, and the encoded amino acid sequence was compared with that in previous reports. The enzyme was characterized according to the optimal pH, temperature and substrate specificity after the expression in *Escherichia coli*.

Materials and methods

Bacterial strains, plasmids and culture conditions

Klebsiella pneumoniae XJ-Li was used as the source of *bud*C gene. *Escherichia coli* BL21 (DE3) pLys was selected for the expression host. Plasmid pET-28a (+) (Novagen) was used for the expression of *meso*-2,3-BDH. The strains were grown in Luria–Bertani (LB) medium at 37 °C.

Construction of expression plasmid for *bud*C gene

The *bud*C gene encoding *meso*-2,3-BDH was amplified via PCR using *K. pneumoniae* XJ-Li genomic DNA as the template. The primers used in PCR were as follows: upstream primer 5′-CATGAATTCATGAAAAAAGTCGCACTTGTTACCG-3′ and downstream primer 5′-CTAAGCTTTTAGTTAAATACCATCCCGCCGTC-3′. The PCR conditions were as follows: 30 cycles of 30 s at 94 °C, 30 s at 63 °C and 1 min at 72 °C after denaturation for 4 min at 94 °C. The amplified product was ligated into the vector pET-28a (+) at *Eco*RI and *Hin*dIII sites, resulting in recombinant plasmid designated as pET-28a (+)-*bud*C. The recombinant *E. coli* BL21 (DE3) pLys/pET-28a (+)-*bud*C was obtained using heat shock transformation.

Preparation and purification of *meso*-2,3-BDH

The transformant containing plasmid pET-28a (+)-*bud*C was grown at 37 °C in LB medium. The *meso*-2,3-BDH was induced with 0.2–1 mM IPTG. Cells were harvested by centrifugation, and the supernatant was then stored. The precipitate was resuspended in 10 mM potassium phosphate buffer (pH 8.0) and disrupted through utralsonication for 10 min. The sample was then centrifuged. The supernatant and pellet were analyzed via SDS-PAGE.

Meso-2,3-BDH was initially purified using an inclusion body protein purification kit (BeiJing Cowin Biotechnology Corp, Ltd, China). After dissolving the pellets in buffer A (20 mM Tris/HCl at pH 7.9, 10 mM imidazole and 0.5 M NaCl), Ni-nitrilotriacetate agarose gel column was used to further purity enzyme with buffer B (20 mM Tris/HCl at pH 7.9, 500 mM imidazole, 0.5 M NaCl and 8 M urea) as the eluent. An on-column purification and refolding method was finally used to prepare the active *meso*-2,3-BDH (Yin et al. 2003).

Enzyme assays

Enzyme activities were determined using either 100 mM polyhydric alcohols with 5 mM NAD^+ or 50 mM ketones with 5 mM NAD^+ and following the changes in absorbance at 340 nm (González et al. 2000). One unit of activity corresponds to 1 mmol NAD(H) formed per min.

Results and discussion

Cloning and sequence analysis of the *meso*-2,3-BDH gene

The *bud*C gene with the Genbank Accession number JN865245 was obtained by PCR. The amino acid identity between the hypothetical protein and other 2,3-butanediol dehydrogenases was high, ranging from 47 to 98 % (Table 1). *Meso*-2,3-BDH from *K. pneumoniae* XJ-Li exhibited a unique molecular weight and amino acid sequence although it had a significant level of identity with that from *K. pneumoniae* and *K. variicola*. However, like other 2,3-butanediol dehydrogenases, except that from *Saccharomonospora viridis*, hypothetical protein also contained three conserved regions: GHGGKIINA, PGIVKTPM and RLSEPEDVAA. Different amino acid sequence of 2,3-BDH from *S. viridis* may be attributed to a Zn-containing enzyme of the medium-chain dehydrogenases/reductases

Table 1 Alignment of the sequence from the *bud*C gene of *K. pneumoniae* XJ-Li

Organism	Gene[a]		Protein[a]	
	Length (bp)	Identity (%)	Molecular mass (Da)	Amino acid identity (%)
K. pneumoniae XJ-Li	771	100	26,635	100
K. pneumoniae	771	97	26,543	98
Klebsiella variicola	771	92	26,533	97
Enterobacter sp. 638	771	79	26,736	89
Bacillus licheniformis	783	63	27,185	66
Lactobacillus buchneri	774	59	26,718	61
Saccharomonospora viridis	789	55	27,896	47

[a] The gene sequences used for the *bud*C gene homologous analysis are from GenBank databank, and their deduced amino acids sequences were used to perform the protein identities. Accession numbers of the gene sequence are NC011283 (*K. pneumoniae*), NC013850 (*Klebsiella variicola*), NC009436 (*Enterobacter* sp. 638), NC006270 (*Bacillus licheniformis*), NC015428 (*Lactobacillus buchneri*) and NC013159 (*Saccharomonospora viridis*)

family (González et al. 2001). In addition, the hypothetical protein contained a NAD^+-binding motif and an active site motif similar to the short chain dehydrogenase/reductase family (SDR family). Such observations indicated that the *bud*C gene may be a potential candidate that encodes 2,3-BDH, and *meso*-2,3-BDH belongs to the short chain SDR family.

Expression and purification of recombinant *meso*-2,3-BDH

The transformant was induced by 0.4 mM IPTG at 25 °C when OD_{600} reached 0.5. The results of preparing *meso*-2,3-BDH are shown in Fig. 1. Only the target protein band was observed in the precipitate, indicating that the recombinant *meso*-2,3-BDH is mainly presented in the intracellular state. The thick protein band representing the pellet from the ultrasonic disruption indicated that the target protein exists mainly in the form of an inclusion body.

The purification of *meso*-2,3-BDH is given in Fig. 2. The inclusion body was purified 1.9-fold (92.8 % purity) using a Ni-nitrilotriacetate agarose gel column. Through on-column purification and refolding, the highest activities were determined as 218 U/mg for reduction of acetoin and 66 U/mg for oxidation of *meso*-2,3-butanediol. This oxidation activity was much higher than that from *E. coli* JM109/pBDO(R) 118 (6.9 U/mg) when 2 % (w/v) glucose was used as an inducer (Ui et al. 1997a). Wardwel et al. (2001) obtained a 440 U/mg reduction activity by expressing *K. pneumoniae* CG21 2,3-BDH gene in *Clostridium acetobutylicum* ATCC 824 driven by clostridial ptb promoter.

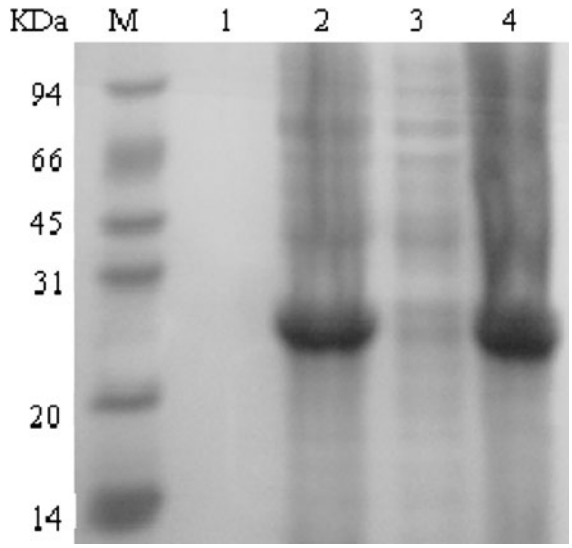

Fig. 1 SDS-PAGE analysis of location and existing state of expression products. *Lane M* protein marker; *lane 1* supernatant of cell culture fluid; *lane 2* cell precipitate centrifuged from cell culture fluid; *lane 3* centrifugal supernatant from utralsonic disruption of cell precipitate; *lane 4* pellet from utralsonic disruption of the cell precipitate

Therefore, the expression level of 2,3-BDH may be affected by various factors, including cultivation conditions, plasmids and promoter types.

Characterization of *meso*-2,3-BDH

As shown in Fig. 3a, *meso*-2,3-BDH was optimal at 35 °C for both reduction and oxidation activities. The optimal pH values were 8.0 and 9.0, respectively (Fig. 3b), which were significantly different from BDH

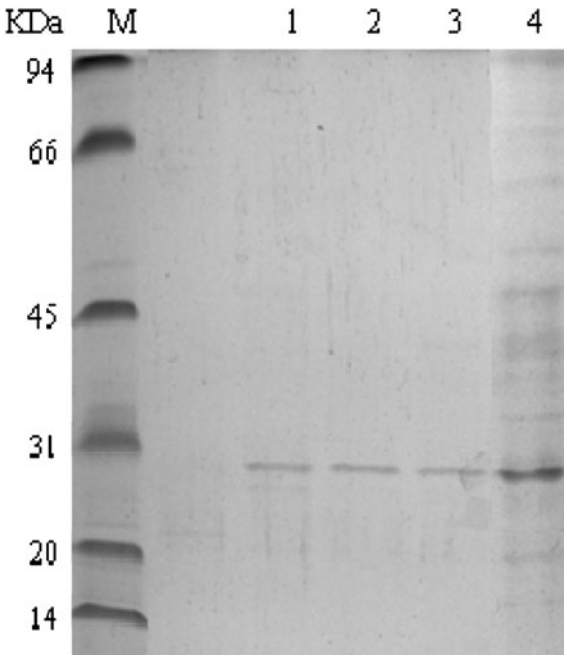

Fig. 2 SDS-PAGE analysis of purified *meso*-2,3-butanediol dehydrogenase. *Lane M* protein marker; *Lane 1* enzyme by on-column purification and refolding; *lane 2* purified enzyme by Ni-nitrilotriacetate agarose gel column; *lane 3* purified enzyme by an inclusion body protein purification kit; *lane 4* dissolved sample (in buffer A) of utralsonic disruption pellet

of *K. pneumoniae* IAM1063 and *Klebsiella terrigena* VTT E-74023. Both butanediol dehydrogenases in these two species exhibited optimal pH values from 5 to 6 in reduction and pH 10–10.5 in oxidation (Ui et al. 1997b). However, the optimal pH for oxidation activity of the BDH from *E. areogenes* 1033 was only 4.4–5.5 (Ui et al. 1997b) and 2,3-butanediol synthesis is generally promoted at low pH levels (i.e. acidic condition) (Celińska and Grajek 2009).

Properties of *meso*-2,3-BDH catalyzing the different substrates

Substrate specificity of *meso*-2,3-BDH is given in Table 2. The enzyme did not exhibit any significant activity toward 2*R*,3*R*-2,3-butanediol, 1,4-butanediol or glycerol. The K_m for NADH was 7.6 % of that for NAD^+, which was similar as described in previous studies (Ui et al. 1997b; González et al. 2000, 2001). The high affinity for acetoin indicated that *meso*-2,3-BDH can easily reduce acetoin to *meso*-2,3-butanediol. Consequently, *meso*-2,3-BDH shows a high stereospecificity, suggesting that *meso*-2,3-BDH can be potentially used in synthesizing chiral acetoinic pure compounds.

Conclusion

The key reaction of the fermentative breakdown of acetoin involves the acetoin dehydrogenase system. Reducing acetoin to 2,3-butanediol requires 2,3-BDH. *Meso*-2,3-BDH was cloned and expressed in *E. coli* BL21 (DE3) pLys, sharing more than 47 % amino acid sequence identity with other known 2,3-butanediol dehydrogenases. After purification and refolding the inclusion body enzyme, 218 and 66 U/mg of reduction

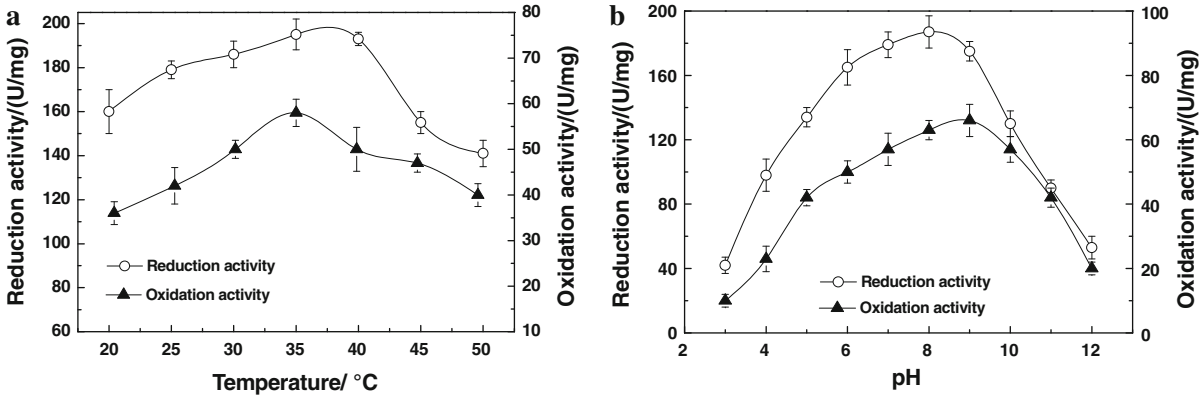

Fig. 3 Optimal temperature and pH of *meso*-2,3-butanediol dehydrogenase. **a** Optimal temperature was determined at pH 8.0 using purified and refolded *meso*-2,3-butanediol dehydrogenase. **b** Optimum pH was determined at 35 °C using purified and refolded *meso*-2,3-butanediol dehydrogenase. Oxidation activities were measured using 100 mM *meso*-2,3-butanediol as substrate with 5 mM NAD^+. Reduction activities were measured using 50 mM acetoin as substrate with 0.5 mM NADH. The values represent the means of three independent experiments (mean ± standard error)

Table 2 Substrate specificity of *meso*-2,3-butanediol dehydrogenase

Substrate	Special activity (U/mg)	K_m (mM)
Oxidation		
meso-2,3-Butanediol	53 ± 0.4	13 ± 0.32
2S,3S-2,3-Butanediol	2 ± 0.2	ND[b]
2R,3R-2,3-Butanediol	<0.1[a]	
1,4-Butanediol	<0.1[a]	
1,3-Propanediol	2 ± 0.1	ND[b]
Glycerol	<0.1[a]	
Reduction		
Acetoin	168 ± 0.5	0.65 ± 0.11
3-Hydroxy-3-methyl-2-butanone	9 ± 0.2	ND[b]
4-Methyl-2-pentanone	21 ± 0.3	6 ± 0.56
Cofactor		
NADH		0.05 ± 0.01[c]
NAD^+		0.66 ± 0.05[c]

[a] Special activity was given as <0.1 while the *meso*-2,3-BDH activity was not detected

[b] ND indicates that the K_m value of the *meso*-2,3-BDH was not determined because of lower enzyme activities

[c] K_m values for NAD^+ and NADH of *meso*-2,3-BDH were determined using the reaction system with 120 mM *meso*-2,3-butanediol and 50 mM acetoin, respectively. Data are means ± standard deviations from three replicates

and oxidation activities were respectively obtained. *Meso*-2,3-BDH also exhibits resistance to alkaline environments. This feature provides a basis for building a new catalysis system. The higher affinity of *meso*-2,3-BDH to acetoin indicates that *meso*-2,3-BDH can easily reduce acetoin to *meso*-2,3- butanediol. The high stereospecificity of the enzyme may make it be potentially used in synthesizing chiral acetoinic pure compounds.

Acknowledgments This work was supported by grants from by the National Natural Science Foundation of China (21104047).

References

Celińska E, Grajek W (2009) Biotechnological production of 2,3-butanediol-current state and prospects. Biotechnol Adv 27:715–725

Converti A, Perego P, Del Borghi M (2003) Effect of specific oxygen uptake rate on *Enterobacter aerogenes* energetics: carbon and reduction degree balances in batch cultivations. Biotechnol Bioeng 82:370–733

González E, Fernández MR, Larroy C et al (2000) Characterization of a (2*R*,3*R*)-2,3-butanediol dehydrogenase as the *Saccharomyces cerevisiae* YAL060W gene product. J Biol Chem 275(46):35876–35885

González E, Fernández MR, Larroy C et al (2001) Characterization and functional role of *Saccharomyces cerevisiae* 2,3-butanediol dehydrogenase. Chem Biol Interact 130–132: 425–434

Ji XJ, Huang H, Li S et al (2008) Enhanced 2,3-butanediol production by altering the mixed acid fermentation pathway in *Klebsiella oxytoca*. Biotechnol Lett 30:731–734

Ma C, Wang A, Qin J et al (2009) Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol 82:49–57

Nicholson WL (2008) The *Bacillus subtilis* ydjL (*bdhA*) gene encodes acetoin reductase/2,3-butanediol dehydrogenase. Appl Environ Microbiol 74(22):6832–68388

Ui S, Okajima Y, Mimura A et al (1997a) Molecular generation of an *Escherichia coli* strain producing only the *meso*-isomer of 2,3-butanediol. J Ferment Bioeng 84(3):185–189

Ui S, Okajima Y, Mimura A et al (1997b) Sequence analysis of the gene for and characterization of D-acetoin forming *meso*-2,3-butanediol dehydrogenase of *Klebsiella pneumoniae* expressed in *Escherichia coli*. J Ferment Bioeng 83(1):32–37

Ui S, Mimura A, Ohkuma M, Kudo T (1999) Formation of chiral acetoinic compound from diacetyl by *Escherichia coli* expressing meso-2,3-butanediol dehydrogenase. Lett Appl Microbiol 28:457–460

Ui S, Takusagawa Y, Sato T et al (2004) Production of L-2,3-butanediol by a new pathway constructed in *Escherichia coli*. Lett Appl Microbiol 39:533–537

Van Houdt R, Aertsen A, Michiels CW (2007) Quorum-sensing-dependent switch to butanediol fermentation prevents lethal medium acidification in *Aeromonas hydrophila* AH-1 N. Res Microbiol 158:379–385

Wardwell SA, Yang YT, Chang HY et al (2001) Expression of the *Klebsiella pneumoniae CG21* acetoin reductase gene in *Clostridium acetobutylicum* ATCC 824. J Ind Microbiol Biotechnol 27:220–227

Yin SM, Zheng Y, Tien P (2003) On-column purification and refolding of recombinant bovine prion protein: using its octarepeat sequences as a natural affinity tag. Protein Expres Purif 32:104–109

Zhang GL, Jiang YL, Ban LL (2011) Process optimization for producing 2,3-butanediol by fermentation of cotton stalk hydrolysate after detoxification. Trans CSAE 27(5): 287–291 (in Chinese with English abstract)