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RESEARCH PAPER

Cloning and Expression of the Genes Encoding Glycerol Dehydratase Reactivase and Identification of Its Biological Activity

LI Wen-Jun, FANG Bai-Shan*, HONG Yan, WANG Xiao-Xia, LIN Jin-Xia, LIU Gui-Lan

Key Laboratory of Industrial Biotechnology, Hua Qiao University), Quanzhou 362021, China

Abstract: The *gdr*A and *gdr*B genes encoding glycerol dehydratase reactivase were amplified using the genomic DNA of *Klebsiella pneumoniae* as the template. The *gdr*A and *gdr*B genes were inserted into pMD-18T to yield the recombinant cloning vector pMD-*gdr*AB. After the DNA sequence was determined, the *gdr*AB gene was subcloned into expression vector pET-28a(+) to yield the recombinant expression vector pET-28*gdr*AB. Under screening pressure by ampicillin and kanamycin simultaneously, the activity of glycerol dehydratase reactivase was characterized by the coexpression of pET-32*gld*ABC, which carried the *gld*ABC gene encoding glycerol dehydratase, and pET-28*gdr*AB in *E. coli* BL21 (DE3).

Key Words: glycerol dehydratase; glycerol dehydratase reactivase; coexpression; incompatible plasmids; molecular chaperone

3-Hydroxypropionaldehyde (3-HPA) has several significant industrial applications. It can be used as a food preservative, as a precursor for the synthesis of many modern chemicals such as acrolein, acrylic acid, and 1,3-propanediol (1,3-PD), and for the synthesis of polymers[1,2]. To date, 3-HPA can be mainly obtained through hydration technology of acrolein[3] and hydroformylation of ethylene oxide[4]; however, these techniques have several disadvantages. Nowadays, the microbiological fermentation method for yielding 3-HPA has attracted the attention of several researchers.

Glycerol dehydratase (GDHt; EC 4.2.1.30) catalyzes the rate-limiting step in the anaerobic conversion of glycerol to 1,3-PD. During the coenzyme B_{12}-dependent catalysis, the enzyme undergoes irreversible inactivation by glycerol or oxygen. Inactivation involves irreversible cleavage of the Co–C bond of coenzyme B_{12}, forming 5′-deoxyadenosine and an alkylcobalamin-like species, which tightly bind to the GDHt's active site, thereby resulting in severe limitation of the organism's ability to ferment glycerol[5]. However, the recent studies have indicated that two homologous open reading frames (*gdr*A and *gdr*B) for the GDHt of *Klebsiella pneumoniae* were proposed to be involved in the reactivation of GDHt. They named the gene products glycerol dehydratase reactivase, which rapidly reactivated glycerol-inactivated or O_2-inactivated GDHt in the presence of ATP, Mg^{2+}, and coenzyme B_{12}[6–9]. According to the analysis of the crystal structure of reactivase, Liao DI proposed 'subunit swap' hypothesis to interpret the mechanism of reactivation[10]. The glycerol dehydratase reactivase has been an attractive component for the study on microbiological fermentation.

Generally, two incompatible plasmids cannot be used in the coexpression of recombinant protein in *E. coli* because of their rapid loss during cell growth and distribution[11]. However, this drawback can be overcome when two kinds of antibiotic

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* Corresponding author. Tel: +86-595-2691560; E-mail: fangbs@hqu.edu.cn
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are present in the selective medium[12,13]. In the present study, the *gdr*A and *gdr*B genes have been amplified using the genomic DNA of *Klebsiella pneumoniae* as the template, and the expression plasmid has been constructed. Moreover, the activity of glycerol dehydratase reactivase was characterized by coexpression of two incompatible plasmids in *E. coli* BL21 (DE3). The use of the two incompatible plasmids in the coexpression of recombinant protein in *E. coli* under screening pressure by ampicillin and kanamycin simultaneously has been approved.

1 Materials and methods

1.1 Materials

1.1.1 Bacterial strains and plasmids: The overexpression vector, pET-32*gld*ABC, which was constructed in our laboratory , was used for the expression of the *gld*ABC (GDHt) genes. pMD18-T and pET-28a(+) were preserved in our laboratory. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as hosts for the cloning and expression experiments. *Klebsiella pneumoniae* DSM2026 was provided by Dr. Zheng AP (German Research Center for Biotechnology).

1.1.2 Enzyme tool and reagents: T4 DNA ligase, restriction endonucleases, and rTaq DNA polymerase were obtained from TaKaRa Biotechnology (Dalian, China) Co Ltd. E.Z.N.A. Gel extraction Kit, E.Z.N.A. Plasmid Miniprep Kit and ATP were provided by Xiamen Tagene Biotechnology Ltd, China. Coenzyme B_{12} and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were from SIGMA Corporation, USA. Other chemical reagents were analytically pure and were purchased from China.

1.1.3 Media and growth conditions: *E. coli* was routinely grown at 37 °C in Luria-Bertani medium, supplemented with ampicillin (100 mg/L) and kanamycin (50 mg/L) whenever necessary.

1.1.4 Primers (synthesized by Peking AuGCT Biotechnology Ltd, China): According to the sequences of *dha*B_4 and *orf*2b from *K. pneumoniae* (ATCC25955) (GenBank accession number U30903), two pairs of primers were designed in this study. Primers for amplifying *gdr*A were as follows (without restriction recognition sites but including ribosome recombinant site):

Primer I:

5′-ATGCGGAGGTCAGCATGCCGTTAATAG-3′;

Primer II:

5′-AGATTAGCCTGACCAGCCAGTAGCAGC-3′;

Primers for amplifying *gdr*B were (including *Eco*R I and *Sma* I respectively):

Primer III: 5′-CCGGAATTCTCGCTTTCACCGCCA-3′;

Primer IV: 5′-TCCCCCGGGTCAATTTCTCTCACT-3′.

1.2 Methods

1.2.1 PCR amplifying *gdr*A and *gdr*B: The PCR programs for amplifying *gdr*A was: predenaturation at 94 °C for 5 min, denaturation at 94 °C for 50 s, annealing at 57 °C for 45 s, extension at 72 °C for 2 min (30 cycles), and final extension at 72 °C for 10 min. Programs for amplifying *gdr*B was: predenaturation at 94 °C for 5 min, denaturation at 94 °C for 50 s, annealing at 55 °C for 45 s, extension at 72 °C for 50 s (30 cycles), and final extension at 72 °C for 10 min.

1.2.2 Construction of the cloning vector pMD-*gdr*AB: *Gdr*A was cloned into pMD-18T vector to construct pMD-*gdr*A by TA cloning. *Gdr*B was double digested with *Eco*R I and *Sma* I, and inserted into pMD-*gdr*A, which had been linearized with the same enzymes to construct the cloning vector pMD-*gdr*AB. The positive clones identified by restriction enzyme digestion analysis were subjected to DNA sequence analysis (AuGCT Biotechnology Ltd, Peking, China).

1.2.3 Construction of the expression vector pET-28*gdr*AB: After pMD-*gdr*AB was double digested with *Eco*R I and *Hin*d III, the *gdr*AB gene was isolated and inserted into pET-28a(+), which had been digested with the same enzymes. The positive clones identified by restriction enzyme digestion were transformed into host bacterium *E. coli* BL21 (DE3) for expression.

1.2.4 SDS-PAGE: Pellets of cells were suspended in 1× loading buffer and heated to 100 °C for 3 min and were analyzed using 15 % gels of SDS-PAGE electrophoresis with Coomassie Brilliant Blue R-250 stain. The expression products were compared with or without inducing IPTG in *E .coli* BL21 (DE3), respectively .

1.2.5 Expression of recombinant vectors in *E. coli* BL21 (DE3): Equal concentration of pET-28*gdr*AB and pET-32*gld*ABC were cotransformed into *E. coli* BL21 (DE3). The recombinant *E. coli* was selected in Luria-Bertani medium containing 100 mg/L ampicillin and 50 mg/L kanamycin, and was then grown with appropriate antibiotics at 37 °C. A total of 1mmol/L IPTG was added to the culture when OD_{600} reached approximately 0.4. The cultures were incubated for 5 h at 25 °C, and then the cells were collected by centrifugation.

1.2.6 Aether treatment: Cells harvested in the late exponential phase were washed twice with 0.05 mol/L potassium phosphate buffer (pH 8.0) and suspended in the same buffer to a concentration of 0.1 g/mL. Aether (final concentration, 0.5 % [*V*/*V*]) was added into the cell suspension, and the mixture was vigorously shaken for 1 min at room temperature. The cells were then collected by centrifugation, washed twice with 0.05 mol/L potassium phosphate buffer (pH 8.0), and then suspended in the same buffer to a concentration of 0.05 g/mL.

1.2.7 Assays of GDHt and reactivase: The activity of the in situ GDHt was assayed by the MBTH method of Toraya *et al*[14]. This method was based on the ability of aldehydes to react with MBTH, forming the azine derivatives, which can be determined spectrophotometrically. The usual assay mixture