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HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE FROM THE BASIODIOMYCETE *PHOLIOTA NAMEKO*

YAN LI, JIA NING WAN, YUKIKO KINJO, NORIHIRO SHIMOMURA, TADANORI AIMI*
Faculty of Agriculture,Tottori University,
Koyamacho Minami 4-101, Tottori-Shi, Tottori, 680-8553,
Japan.
liyan.caas@gmail.com

ABSTRACT

The gene encoding mannitol-1-phosphate dehydrogenase, *mpd*, which has similar function with alcohol dehydrogenase, has been sequenced and characterized from the basiodiomycete *Pholiota nameko*. The coding region of *mpd* was composed of 2992 bp and found to encode a polypeptide of 359 amino acids that has similarity with *Laccaria bicolor*. To evaluate the expression level of mannitol-1-phosphate from *P. nameko*, *mpd* cDNA was inserted into pCold shock vector and expressed in host BL21 (DE3) by 24 h induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 15 ^{O}C after 2 h growth at 37 ^{O}C. The purified protein was detected by SDS-PAGE and western blot.

Keywords: Mannitol-1-phosphate dehydrogenase; Alcohol dehydrogenase activity

INTRODUCTION

Ethanol as a petroleum substitute has many desirable features that are compatible with sustainable development. Most ethanol is produced in large quantities from natural resources including corn grain and sugarcane juice. However, there is a need to find an inexpensive and widely available source of lignocellulosic biomass. It is well-known that basidiomycete fungi cause white rot decay, and are able to degrade the lignin in wood and convert cellulose into glucose which is the most desirable sugar for fermentation.

According to Kalavati [1], mannitol-1-phophate dehydrogenase from *Crytococcus neoformans* is a zinc-containing long-chain alcohol dehydrogenase. Mannitol is a six carbon polyol which is abundant in plants, fungi and bacteria. It accumulates in some fungi and may be involved in fungal tolerance to environmental stress such as salt stress, and may also function as an osmoregulatory compound in supporting turgor and in fruit body development. Mannitol-1-phosphate dehydrogenase catalyzes the conversion of fructose-6-phosphate to mannitol-1-phosphate, which plays an important role in mannitol biosynthesis. Based on the similarity between mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase, we have attempted to mutate the conserved domain in mannitol-1-phosphate dehydrogenase from *P. nameko*, and have investigated the alcohol-producing potential of the mushroom.

MATERIALS AND METHODS

Fungal strain and plasmids *P. nameko* NGW19-6 (*A4, pdx1*), an auxotrophic mutant for pyridoxine, was used in this study [19, 20]. *Escherichia coli*, strain DH5α, (Takara Bio, Shiga, Japan) was used as the host for plasmid amplification. Plasmid pMD20 vector (Takara Bio) was used to clone polymerase chain reaction (PCR) products. *E. coli*, strain BL21, (DE3) (Novagen, Darmstadt, Germany) was used as the host for recombinant protein gene expression, and pCold (Novagen, Darmstadt, Germany) vector was used to clone and express the recombinant protein gene.

DNA and RNA preparation. *P. nameko* mycelium was grown on potato dextrose agar (PDA) at 25 ^{O}C for 7 d. After harvesting, the mycelium was frozen in liquid nitrogen and ground in a mortar and pestle to a fine power. The genomic DNA extraction kit (Amersham Bioscience, Tokyo, Japan) was used to isolate genomic DNA according to the manufacturer's instructions. RNeasy mini kit (Qiagen, Tokyo, Japan) was used to extract RNA.

Genome walking. Genomic DNA from *P. nameko* NGW19-6 was digested with restriction endonucleases (Bgl, Xho, EcoT221, Sal, Xba, Bam, Pst, Hind, Nhe, EcoRI, Fba, Spe) and fragments of suitable length were ligated with nucleotide linkers and used as templates for PCR. PCR was carried out in 100 µl reaction mixtures containing 1 × Ex *Taq* buffer, 100 ng extracted genomic DNA, 100 pmol of each primer, 0.2 mM dNTPs, and 2.5 U Ex *Taq* polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94 ^{O}C for 1 min, followed by 30 cycles of 30 s at 94 ^{O}C and 5 min at 68 ^{O}C. All amplified DNA fragments were subcloned into T-vector pMD20 and all plasmids were sequenced. In order to obtain the full sequence of the mpd gene, after genomic walking, four primers were designed for partial sequencing. PCR was carried out in 50 µl reaction mixtures containing 1 × Ex *Taq* buffer; 100 ng extracted genomic DNA, 100 pmol of each primer, dNTPs at a final concentration of 0.2 mM, and 2.5 U Ex *Taq* polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94 ^{O}C for 5 min, followed by 30 cycles of 30 s at 94 ^{O}C, 30 s at 55 ^{O}C, and 5 min at 72 ^{O}C, followed by 72 ^{O}C for 10 min.

Reverse transcription-PCR (RT-PCR). Amplification of 3'-ends of cDNA by 3'-rapid amplification of cDNA ends (RACE) was performed with a Takara RNA PCR Kit (AMV) Version 3.0 (Takara Bio). The 5'RACE was carried out with the 5'-Full RACE Core Set (Takara Bio). Reverse transcription and PCR were carried out according to the manufacturer's instructions.

DNA sequencing and computer analysis. All the fragments were cloned into T-vector pMD20 and sequenced by ABI PRISM™ 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator™ Cycle Sequencing Kit (version 3.1; Applied Biosystems) according to the manufacturer's instructions. Sequence data editing and assembling were performed using Seqman (DNAStar, Lasergene software, Madison, USA); the subcellular location of MPD was predicted by the program SOSUI [2]; the motif search program online (http://motif.genome.jp/) was used for motif searching. Amino acid sequences of 26 fungal mannitol-1-phosphate and 12 alcohol dehydrogenase amino acid sequences were downloaded from the NCBI at the NIH (http://www.ncbi.nlm.nih.gov/). Initially, all the amino acid sequences were aligned with the Clustal X program [3]. Then, the PHYLIP 3.69 package [4] was used to construct a Jones-Taylor-Thornton matrix from the raw data

(PRODIST program) [5]. Cluster analysis was performed using the unweighted pair group method with arithmetic averaging (UPGMA) [6]. The robustness of the phylogenetic trees was evaluated by bootstrap analysis with 100 replicates [7].

Expression of mannitol-1-phosphate dehydrogenase cDNA in *E. coli*. For expression of MPD cDNA in *E. coli*, a DNA fragment containing the MPD coding region, framed with restriction enzyme sites at both ends, was obtained by PCR with the sequence specific primers designed based on the restriction enzyme sites *Nde*I and *Eco*RI. The PCR fragment was digested with *Nde*I and *Eco*RI and ligated with *Nde*I/*Eco*RI-digested pCold shock vector; this generated plasmid pCold-MPD, which was used to transform BL21 (DE3). The empty vector pCold was used as control. Both plasmid and control were grown at 37 ^{O}C in LB medium supplemented with 100 mg/ml ampicillin until the OD_{600} reached between 0.4-0.6. The culture was adjusted to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further cultivated for 16 h at 15 ^{O}C. The cells were harvested by centrifugation at 2,600g for 10 min, resuspended in Tris-HCl buffer (pH 7.5), and disrupted by ultrasound. Following centrifugation at 2,600 g for 10 min, the supernatant was used for enzyme assays [8-10]. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol of NADH in 1 min under the reaction conditions.

RESULTS AND DISCUSSION

The nucleotide sequence of the mannitol-1-phosphate dehydrogenase gene in *P. nameko* contains 2992 bp, and the coding region (from ATG to the stop codon) is 1363 bp and encodes 359 amino acids. The locations of the exons and introns in the gene were determined from the nucleotide sequences of the RT-PCR products. All the introns started with GT and ended with AG. The coding region contained six exons and five introns. In addition, the SOSUI program predicted that the MPD protein was a soluble protein, which was located in the cytoplasm. The motif search program online revealed that MPD has a zinc-containing alcohol dehydrogenase motif from 1916 bp to 2011 bp. A phylogenetic tree was constructed using the deduced amino acid sequences of MPD and 27 mannitol-1-phosphate dehydrogenase proteins and 11 alcohol dehydrogenase proteins. *P. nameko* MPD protein had a 73% amino acid identity with MPDH1 of *Laccaria bicolor* [11]. The phylogenetic tree separated into two main parts, one consisting entirely of mannitol-1-phosphate dehydrogenases and the other a mixture of alcohol dehydrogenases and mannitol-1-phosphate dehydrogenases. In the mixed group, ten fungal alcohol dehydrogenases were clustered together, while another alcohol dehydrogenase from *Saccharomyces cerevisiae* clustered with ten fungal mannitol-1-phosphate dehydrogenases. MPD was expressed in cultures of engineered *E. coli* BL21 (DE3), and the crude cell extract had alcohol dehydrogenase activity.

There have been many reports on mannitol-1-phosphate dehydrogenase genes, which mainly focused on mannitol metabolism. These include the proposal that mannitol is involved in an NADPH-producing cycle involving the utilization of NADH and ATP [12]. However, mannitol was still produced by mannitol-1-phosphate dehydrogenase and mannitol dehydrogenase double deletion mutants [13] and the existence of a novel mannitol phosphorylation pathway for mannitol biosynthesis and metabolism was deduced. Mannitol is the main storage material in *Agaricus bisporus* [14] and also plays important roles in the sporulation of *Stagonospora nodorum* [15]. Mannitol accumulation in fungi may be involved in fungal tolerance to environmental stress such as salt stress [16], and function as an

osmoregulatory compound to support turgor and fruit body development [17]. However, there are no reports describing the relationship between mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase. Here, we report for the first time a mannitol-1-phosphate dehydrogenase gene from *P. nameko* that encodes a protein with the potential to produce alcohol by fermentation. The long chain alcohol dehydrogenase from horse liver has approximately 350 amino acids per subunit, and the short chain alcohol dehydrogenase from *Drosophila* has approximately 250 amino acids per subunit [18]. According to our data, mannitol-1-phosphate dehydrogenase from *P. nameko* has 359 amino acids and is a zinc-containing long chain alcohol dehydrogenase.

Phylogenetic analysis suggests that mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase are evolutionary homologous, and some mutations affecting mannitol-1-phosphate dehydrogenase may lead to the appearance of alcohol dehydrogenase. This may explain why some fungi can produce alcohol under anaerobic conditions. Yeasts have the capability of alcoholic fermentation but cannot degrade lignocellulose, while basidiomycetes can degrade lignocellulose but cannot produce alcohol by fermentation. Mannitol-1-phosphate dehydrogenase from *P. nameko* expressed in *E. coli* has alcohol dehydrogenase activity and this makes mushroom alcoholic fermentation possible. Compared with *Agaricus blazei*, *Flammulina velutipes* and *Tricholoma matsutake* [8, 9, 10], the alcohol dehydrogenase activity is very low, but genetic engineering methods such as promoter replacement or mutation could be used to improve alcohol dehydrogenase activity in *P. nameko*.

CONCLUSIONS

Mannitol-1-phophate dehydrogenase from *P. nameko* was cloned and characterized. The presence of the zinc-containing long-chain alcohol dehydrogenase suggests that the mushroom has the potential to produce ethanol by fermentation.

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