J Ind Microbiol Biotechnol (2006) 33:1032–1036
DOI 10.1007/s10295-006-0154-8

ORIGINAL PAPER

Molecular cloning and characterization of the alcohol dehydrogenase ADH1 gene of *Candida utilis* ATCC 9950

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Received: 10 March 2006 / Accepted: 21 June 2006 / Published online: 20 July 2006

Abstract The alcohol dehydrogenase gene (*ADH1*) of *Candida utilis* ATCC9950 was cloned and expressed in recombinant *Escherichia coli*. *C. utilis ADH1* was obtained by PCR amplification of *C. utilis* genomic DNA using two degenerate primers. Amino acid sequence analysis of *C. utilis* ADH1 indicated that it contained a zinc-binding consensus region and a NAD(P)$^+$-binding site, and lacked a mitochondrial targeting peptide. It has a 98 and 73% identity with ADH1s of *C. albicans* and *Saccharomyces cerevisiae*, respectively. Amino acid sequence analysis and enzyme characterization with various aliphatic and branched alcohols suggested that *C. utilis* ADH1 might be a primary alcohol dehydrogenase existing in the cytoplasm and requiring zinc ion and NAD(P)$^+$ for reaction.

Keywords *Candida utilis* · Alcohol dehydrogenase

Introduction

Alcohol dehydrogenase (ADH; EC 1.1.1.1) is a ubiquitous enzyme catalyzing the production and assimilation of alcohols. ADH genes and proteins from yeasts including *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia stipitis* have been sequenced and characterized [1–7]. Few ADH enzymes and genes originating from *Candida* species have been analyzed. Three *ADH* genes encoding a cytoplasmic and two mitochondrial enzymes were cloned from *Candida boidinii*, and proteins that showed both alcohol dehydrogenase activity and methyl formate synthesis activity were reported [8]. A purified methyl formate synthase from *C. boidinii* belonged to the NAD$^+$-dependent class III alcohol dehydrogenase family [9]. The *C. albicans ADH1* gene encoded a 350 amino acid enzyme with high homologies to yeast *ADH*s [10]. Recently, the *ADH2* gene of *C. albicans* was identified by the *C. albicans* genome sequencing project [11]. Purified ADH proteins from *C. utilis* CBS621 were characterized by the determination of optimal reaction conditions and substrate preference with various alcohols [12]. A cell-free extract probably containing *C. utilis* ADH proteins was responsible for ethyl ester production [13]. Nucleotide sequence information or polypeptide sequence of *C. utilis* ADHs have not been published to date.

In this study, the isolation and sequence characterization of the *C. utilis ADH1* gene was carried out. Cofactor preference and substrate specificity with various aliphatic and branched alcohols were investigated by using *C. utilis* ADH1 expressed in recombinant *Escherichia coli*.

Isolation of C. utilis ADH1 gene

Candida utilis ATCC9950 grown in YPD medium [13] and *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) cultivated in LB broth [14] were used for *ADH1*

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amplification, DNA manipulation and *ADH1* expression, respectively. Two PCR primers were designed on the basis of amino acid sequences of *C. albicans* ADH1, *C. boidinii* ADH1 and *S. cerevisiae* ADH1 in order to amplify *C. utilis ADH1*. Since N- and C-terminal sequences of three yeast ADH1s were highly conserved as shown in Fig. 1, MS(E/M)(Q/S)IP(K/T/E)TQK(A/G)V for N-terminal sequence and GRYV(L/V)DT(S/T)K for C-terminal sequence were used to prepare two degenerate DNA oligomers. Especially, four amino acids [MS(E/M)(Q/S)] were referred to *C. albicans* ADH1 because amino acid sequences of *C. utilis* such as URA3 (accession number: Y12660), ribosomal protein L25 (X05919), isocitrate lyase (AJ404885) and biotin synthase (AF212161) showed a high score of homology to the corresponding *C. albicans* proteins (data not shown). Two primers for the amplification of the *C. utilis ADH1* entire coding region were designed as 5′-ATGWSNRWRVVNATH CCNRMNACNCARAARGSNGTN-3′ for N-terminal and 5′-MMAYTTNSWNGTRTCNAVNACR-TANCKNCC-3′ for C-terminal. DNA amplification was performed by TaKaRa Ex Taq kit (TaKaRa, Otsu, Higa, Japan) using a PCR machine (Robocycler Gradient 96, Stratagene, La Jolla, CA, USA) programmed with a temperature profile: 94°C for 5 min (1 cycle); 94°C for 1 min, 52°C for 1 min and 72°C for 70 s (30 cycles); 72°C for 5 min (1 cycle). The genomic DNA of *C. utilis* collected by a standard method [23] was used as a PCR template. A thick and bright DNA band of 1.0 kb size on 1% agarose gel was collected and purified with the Gel extraction kit (Qiagen Inc., Valencia, CA, USA). Purified DNA was ligated with plasmid pCR2.1-TOPO (Invitrogen, USA) and its product, plasmid pYADH5, was transformed into *E. coli* TOP10 chemical competent cells. Seventeen small colonies exhibited white color on LB broth plate with 50 mg/l ampicillin, 2% agar and 40 mg/l 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside. After the extraction of plasmids from the transformants, *Eco*RI digestion allowed five clones to be chosen for DNA sequencing by LS Labs Inc. (Houston, TX, USA).

Sequence analysis of *C. utilis* ADH1

DNA sequences from five clones were identical and contained 1,053 bp of the *C. utilis ADH1* gene with a 43.6% GC content. The nucleotide sequence of *C. utilis ADH1* was analyzed with the discontiguous Mega BLAST program (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST]) using the default parameters, resulting in a strong homology to *ADH1* (X81694, 97% identity) and *ADH2* (XM_712482, 85%) of *C. albicans* [10, 11]. The 1,022 nucleotides of *C. utilis ADH1* overlapped with *C. albicans ADH1*. In addition, this gene was highly similar to other yeast *ADH* genes: *ADH1* (AB125900, 80%), *ADH2* (AB125901, 78%) and *ADH3* (AB125902, 78%) of *C. boidinii* [8]; *ADH1* (AF008245, 76%) and *ADH2* (AF008244, 76%) of *P. stipitis* [2]; *ADH1* (M38456, 76%) and *ADH2* (J01314, 79%) of *S. cerevisiae* [1, 4]. The standard translation codons were used to obtain the polypeptide sequence of *C. utilis* ADH1 of 350 amino acids. The sequence alignment with yeast ADHs by the CLUSTALW program at the Biology Workbench (version 3.2; the San Diego Supercomputer Center, the University of California, San Diego [http://www.workbench.sdsc.edu]) using the standard parameters is presented in Fig. 1. The theoretical molecular weight and isoelectric point of *C. utilis* ADH1 were estimated at 36,800 and 5.89, respectively. Except for six amino acids such as Thr_2, Asn_{39}, Trp_{53}, Arg_{57}, Ser_{59} and Ala_{222}, the *C. utilis* ADH1 amino acid sequence was the same as that of *C. albicans* ADH1 (98% identity). *C. utilis* ADH1 had a high identity (up to 71%) to ADHs from *P. stipitis*, *C. boidinii*, *S. cerevisiae* and *K. lactis* [1, 2, 5, 8]. The amino acid sequence alignment showed that *C. utilis* ADH1 was deficient in an N-terminal mitochondrial target peptide (Fig. 1, box I). Yeast alcohol dehydrogenases are located in the cytoplasm or mitochondria. Mitochondrial ADHs such as *C. boidinii* ADH2 and ADH3 [8], *S. cerevisiae* ADH3 [7] and *K. lactis* ADH3 [6] possessed 21–25 N-terminal amino acids specific for localization to mitochondrial membrane. Considering the metal binding region of *C. albicans* ADH1 [10] and *C. boidinii* ADH1 and 2 [8], and the GXGXXG fingerprint pattern of the cofactor binding domain [15], *C. utilis* ADH1 contained a zinc-binding consensus (GHEGAGVVCGMGENVK, Box II) and a $NAD(P)^+$ binding motif (GAGGGLG, Box III) as shown in Fig. 1. The $NAD(P)^+$-requiring ADH superfamily can be classified into three groups; Group I, zinc-dependent long chain ADHs (approximately 350 residues per subunit); Group II, zinc-independent short chain ADHs; Group III, iron-activated ADHs [15]. Nucleotide and amino acid sequence analyses suggested that *C. utilis* ADH1 might be a cytoplasmic ADH which is dependent on zinc ion and $NAD(P)^+$, and fit in the ADH superfamily Group I.

Expression and characterization of *C. utilis* ADH1

To express *C. utilis ADH1* in recombinant *E. coli*, the coding region of *C. utilis ADH1* was amplified from plasmid pYADH5 by PCR with two primers; 5′-CG

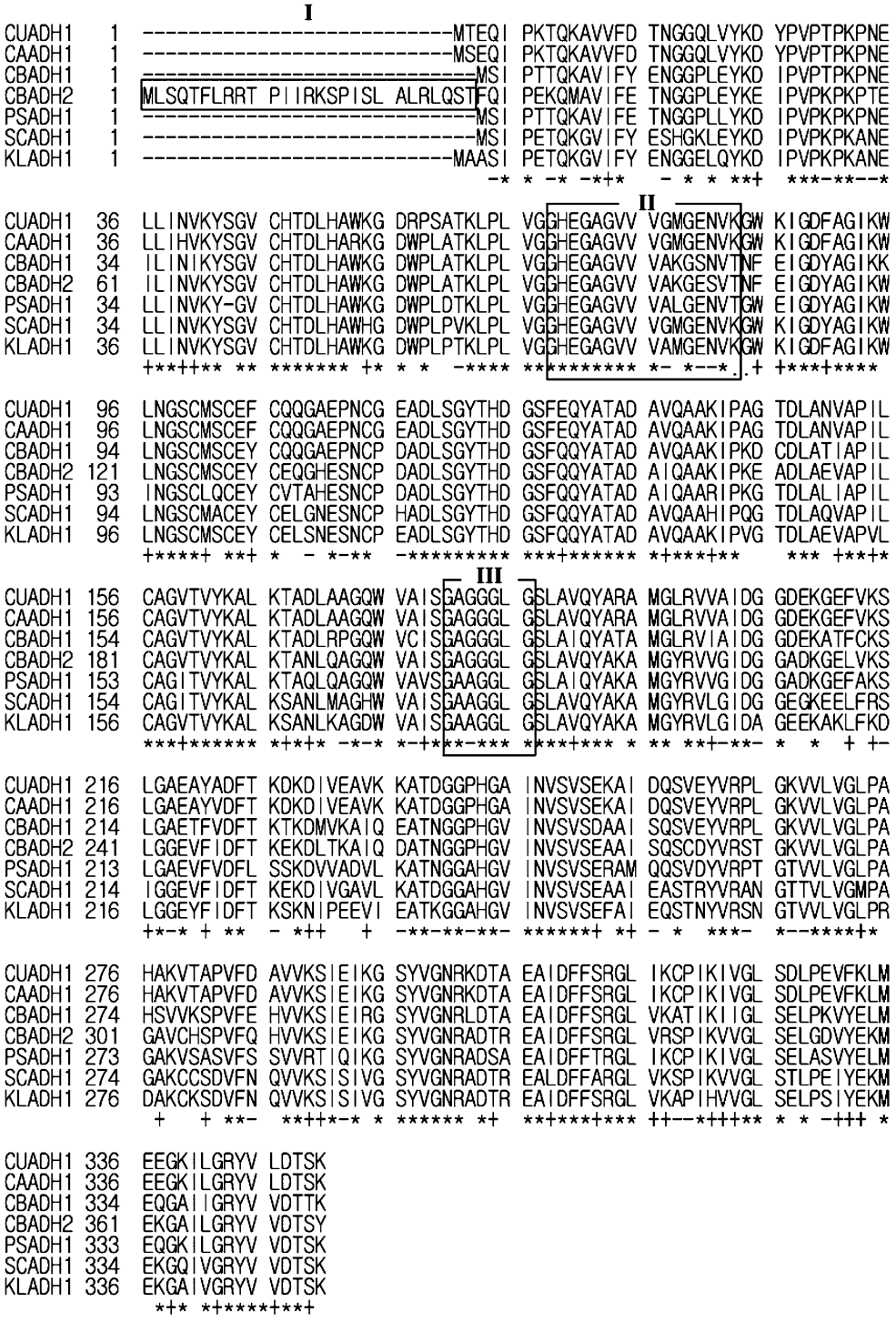

Fig. 1 CLUSTALW alignment of the deduced amino acid sequence of *C. utilis* ADH1 with yeast ADHs. CUADH1, *C. utilis* ADH1 (this study); CAADH1, *C. albicans* ADH1 (accession number, X81694); CBADH1 and CBADH2, *C. boidinii* ADH 1 (AB125900) and 2 (AB125901); PSADH1, *P. stipitis* ADH1 (AF008245); SCADH1, *S. cerevisiae* ADH1 (M38456); KLADH1, *K. lactic* ADH1 (P20369). Box I, mitochondrial targeting region; Box II, zinc-binding consensus; Box III, NAD(P)$^+$-binding moiety. *Symbols* indicate consensus keys as follows; * single, fully conserved residue; + conservation of strong groups; − conservation of weak groups; blank, no consensus

GGATCCATGACGGAACAGATACCTAAG-3′ and 5′-CGGGATCCCTATTTACTGGTGTCCAAGAC-3′. Both contained a *Bam*HI restriction site (underlined). PCR followed the above conditions. PCR-amplified DNA and plasmid pUC19 (Invitrogen, USA) were digested with *Bam*HI and ligated with each other,

leading to the construction of plasmid pUYADH1. Digestion and end filling of plasmid pUYADH1 with *Sal*I and Klenow Fragment were carried out to modify the region between *E. coli* ribosomal binding site and the translational initiation codon of *C. utilis ADH1*. Finally, plasmid pUYADH1m was used for *C. utilis ADH1* expression by the *lac* promoter. To obtain the crude enzyme solution of *C. utilis* ADH1, recombinant *E. coli* TOP10/pUYADH1m was cultured by a shaking incubator (Model G76, New Brunswick Scientific, NJ, USA) in LB medium at 200 rpm and 30°C. At a 0.6 of optical density (600 nm), 1 mM IPTG was added to the culture broth. Since plasmid pUYADH1m in *E. coli* TOP10 cells was unstable and disappeared after long (17 h) IPTG induction (data not shown), cells were harvested after 4 h of induction and pellets were resuspended with 50 mM Tris buffer (pH 8.3). Cells were disrupted by sonication using the Sonicator 3000 (Misonix Inc., Farmingdale, NY, USA) at 4°C and 7.0 output level for 2 min. After centrifugation for 20 min at 4°C, the supernatant was used as a crude enzyme solution. A cell-free solution of *E. coli* TOP10/pUC19 obtained by the same method was used as the control. The reaction with alcohols at 25°C was analyzed by monitoring an absorbance increase at 340 nm caused by NADH formation. Reaction mixture (1 ml) consisted of 50 mM Tris buffer (pH 8.3), 10 mM alcohol, 6 mM $NAD(P)^+$ and crude enzyme solution. One unit was defined as the amount of enzyme to form 1 μmol NAD(P)H per minute at the reaction conditions. Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA, USA). These experiments were done in triplicate. To determine the preference of cofactors, NAD^+ and $NADP^+$ (0.6 mM) were tested in the ADH activity assay with 10 mM ethanol. The specific ADH activity of 14.2 (± 0.4) mU/mg for NAD^+ was 9.0 times higher than that for $NADP^+$ [1.58 (± 0.16) mU/mg], indicating that *C. utilis* ADH1 preferred NAD^+ to $NADP^+$ as a cofactor. Yeast ADHs are usually specific for cofactor NAD(H) [22]. For the modulation of NAD(H) specificity, substitution of Asp to Gly at the 202 position of *S. cerevisiae* ADH1 resulted in low and equivalent kinetic constants for NAD^+ and $NADP^+$, suggesting that even though one point mutation was effective, several substitutions of amino acid residues should be needed to change the cofactor preference without the reduction of ADH activity [16]. Recently, an NADP-dependent alcohol dehydrogenase was purified from *S. cerevisiae* [17]. To investigate the effect of chain length and branch content of alcohols on *C. utilis* ADH1 activity, various primary and secondary alcohols were used in ADH activity analysis (Fig. 2). *C. utilis* ADH1 does not react with methanol. Primary alcohols such as 1-propanol and 1-butanol provided 93–108% relative ADH activities compared with ethanol. 1-Propanol gave the best specific activity of 15.3 (± 0.5) mU/mg. In case of secondary alcohols such as 2-propanol and 2-butanol, 4–5% levels of relative ADH activities compared with ethanol were achieved. *C. utilis* ADH1 reacted slightly with a branched alcohol (isobutanol) and a diol (1,2-butanediol). *S. cerevisiae* ADH1, a primary ADH, displayed a much higher *V*/*K* value on ethanol than those on 2-propanol and 2-butanol [18]. In contrast, *Gordonia* sp. ADH2, a secondary ADH, was fourfold more active on 2-propanol than ethanol [19]. It was reported that purified *C. utilis* ADH enzymes showed high activities with primary alcohols from ethanol to heptanol, and exhibited appreciable activities with secondary alcohols such as 2-propanol and 2-butanol [12]. Meanwhile, upon the previous study showing that *C. utilis* ADH probably catalyzed acetate ester synthesis [13], the crude enzyme of *C. utilis* ADH1 in this study was applied to investigate the ability of ethyl acetate formation from ethanol and acetaldehyde as described by Kusano et al. [20], but no activity in ethyl acetate production was observed.

As a result, the sequence analysis and enzyme characterization of *C. utilis* ADH1 suggested that *C. utilis* ADH1 might be a primary alcohol dehydrogenase present in the cytoplasm and requiring zinc ion and $NAD(P)^+$. In contrast to significant expression levels of the *C. boidinii ADH* genes in *E. coli* [8], low level expression of *C. utilis* ADH1 in recombinant *E. coli* limited its enzymatic characterization. Recombinant expression systems of *C. utilis* or *ADH* deficient *S. cerevisiae* strains might be useful for further characterization of *C. utilis* ADH1 [10, 21]. A previous report of *C. utilis* ADH enzymes mentioned that at least three

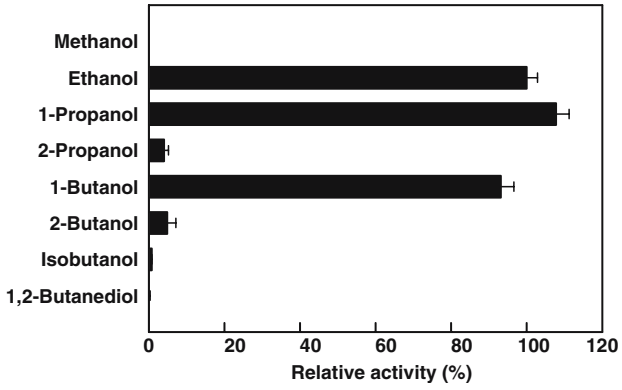

Fig. 2 Relative activity of *C. utilis* ADH1 on various alcohols. Relative activity of 100% corresponded to the specific activity on ethanol

ADH isozymes were present in the purified preparation [12]. The genetic information of *C. utilis ADH1* is helpful for the isolation and analysis of additional *ADH* genes of *C. utilis*. The DNA sequence of *C. utilis ADH1* has been deposited in the NCBI GenBank under Accession Number DQ397054.

Acknowledgment This study was supported by the Korea Research Foundation Grant (KRF-2004-000-10255-0) and USDA-NSF Inter Agency Metabolic Engineering Program: USDA 2002-35505-11638 and NSF BES0118815.

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