Genetics and Molecular Biology, 34, 2, 240-243 (2011)

www.sbg.org.br

Short Communication

Cloning, expression and characterization of alcohol dehydrogenases in the silkworm *Bombyx mori*

Nan Wang, Haifeng Shi, Qin Yao, Yang Zhou, Lequn Kang, Huiqin Chen and Keping Chen

Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu Province, P.R. China.

Abstract

Alcohol dehydrogenases (ADH) are a class of enzymes that catalyze the reversible oxidation of alcohols to corresponding aldehydes or ketones, by using either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), as coenzymes. In this study, a short-chain ADH gene was identified in *Bombyx mori* by 5'-RACE PCR. This is the first time the coding region of BmADH has been cloned, expressed, purified and then characterized. The cDNA fragment encoding the BmADH protein was amplified from a pool of silkworm cDNAs by PCR, and then cloned into *E. coli* expression vector pET-30a(+). The recombinant His-tagged BmADH protein was expressed in *E. coli* BL21 (DE3), and then purified by metal chelating affinity chromatography. The soluble recombinant BmADH, produced at low-growth temperature, was instrumental in catalyzing the ethanol-dependent reduction of NAD^+, thereby indicating ethanol as one of the substrates of BmADH.

Key words: 5'-RACE PCRADH, enzymatic activity, recombinant protein.

Received: September 28, 2010; Accepted: February 17, 2011.

Alcohol dehydrogenases (ADH; EC 1.1.1.1) belong to the oxidoreductase family, a class of enzymes, instrumental in catalyzing the reversible oxidation of alcohols to corresponding aldehydes or ketones, by using either NAD or NADP as coenzymes. ADHs are widely distributed in nature and have been found in species throughout the three domains of life, Archaea, Bacteria and Eukarya (Branden *et al.*, 1975; Reid and Fewson, 1994; Rella *et al.*, 1987). ADHs play important roles in a wide range of physiological processes. Based on their catalytic activities, they presumedly participate in the metabolism of steroids, retinoids, lipid peroxidation products, ω-hydroxy fatty acids, xenobiotic alcohols and aldehydes (Doga, 2010). Based on molecular size and cofactor requirements, ADHs are generally classified into three subfamilies: Type I, the medium-chain zinc-dependent, such as horse liver ADHs and ADHs (isozymes I-III) in *Saccharomyces cerevisiae* (Adolph *et al.*, 2000), contain approximately 370 amino acids per subunit, and form dimers (in higher eukaryotes) or tetramers (in bacteria); Type II, the short-chain zinc-independent ADHs , such as in *Drosophila melanogaster* (Benach *et al.*, 2005), contain approximately 250 amino acids per subunit and, rarely, metals; and Type III, the long-chain iron-activated ADHs , such as ADH IV from *S. cerevisiae* (Williamson and Paquin, 1987), contain 385 to 900 amino acid residues per subunit.

Send correspondence to Keping Chen. Institute of Life Sciences, Jiangsu University, 301# Xuefu Road, Zhenjiang, Jiangsu Province, 212013 P.R. China. E-mail: kpchen@ujs.edu.cn.

In insects, ADHs primarily break down dietary alcohols produced by microbial fermentation (Atrian *et al.*, 1998). A number of short-chain ADH genes have been cloned and characterized from a variety of fruit-fly species, such as *Drosophila melanogaster* (Benach *et al.*, 1999), *Drosophila lebanonensis* (Benach *et al.*, 1999), *Ceratitis capitata* (Mediterranean fruit flies) (Gasperi *et al.*, 1994) and *Bactrocera (Dacus) oleae* (olive fly) (Mazi *et al.*, 1998). Whereas short-chain ADHs from *Drosophila* and certain closely related insects use small alcohols as substrates, all the other known members of this group are mostly steroid and prostaglandin dehydrogenases of both prokaryotic and mammalian origin (Benach *et al.*, 2005). Although much exhaustive biochemical work has been undertaken with *Drosophila* ADHs, few short-chain ADHs from other insects has been functionally characterized.

In this study, the open reading frame (ORF) of putative *Bombyx mori* alcohol dehydrogenases, denominated BmADHs, was cloned, and the recombinant enzymes expressed in *Escherichia coli*. The ethanol dehydrogenase activity of the resultant recombinant proteins was assayed.

An EST, highly similar to *Bombyx mori* ADH cDNA, was identified in the GenBank database (Accession No. rswdd0_001984EST). In order to obtain the full-length cDNA sequence, 5'-RACE was carried out with the SMART RACE cDNA kit (Clontech, CA, USA) by using 1 μg of poly(A)$^+$ mRNA from the posterior silk gland of *B. mori* and a gene-specific reverse (5'-TGT AAA GGA TTG

CAG TGT CAG TGG-3') and forward primer (5'-AAG CAG TGG TAT CAA CGC AGA GT-3') both obtained from Clontech. Specific bands were retrieved, subcloned into pMD-18T vector and identified as a 773 bp cDNA fragment by DNA sequencing. A 1104 bp cDNA sequence containing a complete 822 bp ORF (GenBank accession number DQ512730) encoding a 274 amino acid protein with conserved short-chain ADH domain was obtained from alignment of the obtained fragment and EST.

By comparing the new identified sequence with contigs of *B. mori* genome in GenBank using BLAST and SIM4, five exons and four introns were revealed. The orthologous sequences retrieved from the NCBI database indicated identity of the deduced amino acid sequence of BmADH (GenBank accession no.NP_001037610.1) to be 32%, 31% and 30% with the *Homo sapiens* 15-hydroxyprostaglandin dehydrogenase (Hs15HPGDH), *D. lebanonensis* alcohol dehydrogenase (DlADH) and *D. melanogaster* alcohol dehydrogenase (DmADH), respectively (Figure. 1). On numbering DmADH, the triad of active site residues, Ser139, Tyr152 and Lys156, intimately involved in the enzyme reaction, were found to be conserved in the BmADH protein (Figure. 1).

BmADH specific primers, the forward 5'-GGGGA TCCATGGCACCGGATTTCGTG-3' with a *BamHI* site, and reverse 5'-CCCTCGAGCTATGTCTTGGAGAGTA TTTGGAAG-3' with an *XhoI* site, were designed to amplify the ORF of a putative BmADH gene from a pool of silkworm cDNAs. The tissue expression profile of BmADH was investigated, through RT-PCR analysis of total RNAs isolated from silk glands, malpighian tubules, spermaries, eggs, fat body, ovaries, ganglia, hemocytes, epidermis, hindgut, and midgut of fifth instar larvae. BmADH transcripts were detected in all the examined tissues. The expression levels in hemocytes and the hindgut were higher, when compared to those in other tissues (Figure. 2).

The PCR product, first ligated into the pMD18-T vector using T4 DNA ligase, was then transformed into *E. coli* TG1. A fragment, between *BamHI* and *XholI* and containing the BmADH gene, was excised from the recombinant plasmid. The purified fragment was subcloned into the pET-30a(+) expression vector and transformed into *E. coli* BL21 (DE3). DNA sequencing confirmed correct BmADH ORF fusion to the N-terminal 6xHis tag.

To express recombinant protein, a freshly transformed colony was cultured in LB medium with kanamycin (50 μg/mL) at 37 °C, with vigorous shaking. So as to obtain a soluble protein, on OD_{600} reaching 0.6, BmADH expression was induced with IPTG (final concentration 0.4 mM), and further cultured at 16 °C for 20 h. SDS-PAGE analysis of the *E.coli* lysate revealed that recombinant protein was expressed and the molecular mass was about 31 kDa. The expression of 6xHis-tagged BmADH was confirmed by mouse anti-6xHis monoclonal antibody (1:500 dilution) for 2 h, followed with peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution) for another 2 h to display color, as described previously (Huo *et al.*, 2010).

For purification of recombinant BmADH protein, *E. coli* BL21 cells were harvested by centrifugation (4500 x g, 4 °C, 15 min). The cell pellet was resuspended in buffer A (50 mM sodium phosphate, 300 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, pH 8.0), the resultant cell-suspension then being lysed by sonication. The lysate was clarified by centrifugation (16,000 x g, 4 °C, 25 min). The supernatant was then loaded onto a Ni-NTA affinity column (Qiagen). Purification conditions were standardized by optimizing the pH , the concentrations of salt and imidazole. After washing the captured column with 50 mM imidazole, the fusion protein was eluted with 250 mM imidazole. The eluted protein was dialyzed against buffer B (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) at 4 °C. The concentration of protein was determined by the Bradford

```
DmADH          ------MSFTLTNKNVIFVAGLGGIGLDTSKELLKRDLKNLVILDRIENPAAIAELKAI 53
DlADH          --------MDLTNKNVIFVAALGGIGLDTSRELVKRNLKNFVILDRVENPTALAELKAI 51
BmADH          MAPDFVKRFVDVDDKVFLVTGGAAGVGAGLVKALLFENARHVAFLDVADREGAALEAQLI 60
Hs15HDPGDH     --------MHVNGKVALVTGAAQGIGRAFAEALLLKGAK-VALVDWNLEAGVQCKAALD 50
                       . : .*  :....  *:*    . *: .. : ..::*   .  .  :

DmADH          N--PKVTVTFYPYDVTVPIAETTKLLKTIFAQLKTVDVLINGAGIL--DDHQIERTIAVN 109
DlADH          N--PKVNITFHTYDVTVPVAESKKLLKKIFDQLKTVDILINGAGIL--DDHQIERTIAIN 107
BmADH          IKFGALRVKFIKCDVGD-ERQLASAYKQVLDKYKRLDGVINSAAVLSVDDNSFNRMIDIN 119
Hs15HDPGDH     EQFEPQKTLFIQCDVAD-QQQLRDTFRKVVDHFGRLDILVNNAGVN--NEKNWEKTLQIN 107
                       *   **     : .  : :. :   :* ::*.*. :   :::. :: : :*

DmADH          YTGLVNTTTAILDFWDKRKGGPGGIICNIGSVTGFNAIYQVPVYSGTKAAVVNFTSSLAK 169
DlADH          FTGLVNTTTAILDFWDKRKGGPGGIIANICSVTGFNAIHQVPVYSASKAAVVSFTNSLAK 167
BmADH          FTGTVNSTLKALDIMGADKGGSGGVIVNISSLLALNLTSHLPVYAATKAAVLQFSIRMGT 179
Hs15HDPGDH     LVSVISGTYLGLDYMSKQNGGEGGIIINMSSLAGLMPVAQQPVYCASKHGIVGFTRSAAL 167
                .. :. *   **  .  :** **:* *: *: .:    : ***. .:* . :: *:   .

DmADH          LAPITG--VTAYTVNPGITRTTLVHKFN-----SWLDVEPQVAEKLLAHPTQPSLACAE 221
DlADH          LAPITG--VTAYSINPGITRTPLVHTFN-----SWLDVEPRVAELLLSHPTQTSEQCGQ 219
BmADH          QEQFTRTKVRVLSVCLGPTDTAILYKNNL-----TKFDTEYAPCLSSRAPVRQRVESAVK 234
Hs15HDPGDH     AANLMNSGVRLNAICPGFVNTAILESIEKEENMGQYIEYKDHIKDMIKYYGILDPPLIAN 227
                  :    *   ::  * . *.:: . :       :: :                 :

DmADH          NFVKAIELNQNGAIWKLDLGTLEAIQWTKHWDSGI------ 256
DlADH          NFVKAIEANKNGAIWKLDLGTLEAIEWTKHWDSHI------ 254
BmADH          GILEVINKASTGDTWIVES-DKPAYDFTQNISEAFQILSKT 274
Hs15HDPGDH     GLITLIEDDALNG-AIMKITTSKGIHFQDYDTTPFQAKTQ- 266
               .::  *:    .    :.     . .: .     :
```

Figure 1 - Amino acid sequence alignment of deduced BmADH, *Homo sapiens* 15-hydroxyprostaglandin dehydrogenase (Hs15HPGDH), *D. lebanonensis* alcohol dehydrogenase (DlADH), and *D. melanogaster* alcohol dehydrogenase (DmADH). Accession numbers are NP_001037610.1, P15428, P10807 and P00334, respectively. * indicates identical residues; * indicates enzyme active-center residues; : indicates conserved residues; . indicates semi-conserved residues.

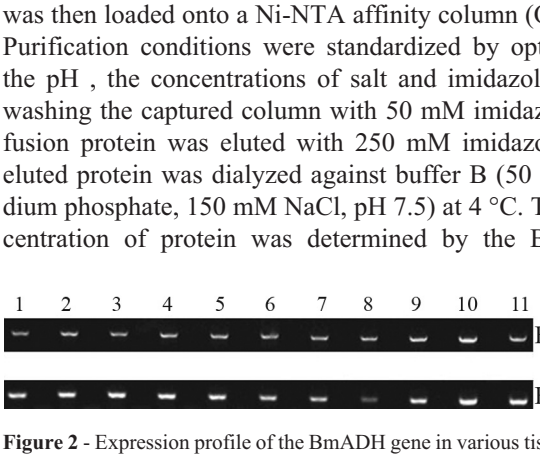

Figure 2 - Expression profile of the BmADH gene in various tissues of *B. mori*. Top: lane1, silk gland; lane 2, malpighian tubule; lane 3, spermarium; lane 4, egg; lane 5, fat body; lane 6, ovary; lane 7, ganglion; lane 8, hemocytes; lane 9, epidermis; lane 10, hindgut; lane 11, midgut. An actin gene of *B. mori* was used as internal control in the corresponding tissues.

method, the estimated purity being over 80%, according to SDS-PAGE analysis (Figure 3A).

The specific bands corresponding to BmADH proteins were analyzed by an ultraflex MALDI-TOF-TOF instrument (Bruker, Germany), whereupon twelve peptide fragments were identified. Peptide mass fingerprinting (PMF) was performed by comparing the masses of identified peptides to those of hypothetical tryptic peptides for proteins in a non-redundant NCBI database, using the MASCOT search engine. BmADH was clearly identified, with a MOWSE score of 86. The sequences of the 12 identified peptide fragments accounted for 45% of amino acid sequences in BmADH.

The catalyzing activity of recombinant BmADH was assayed spectrophotometrically by measuring the increase in absorbance at 340 nm, following the reduction of NAD^+ to NADH in a solution containing substrate ethanol, as described by Oudman *et al.* (1991). Briefly, 3 mL of reaction buffer (50 mM NaOH/Glycine buffer, pH 9.0, 0.67 M ethanol and 8 mM NAD^+) were incubated at 25 °C, and the reaction was initiated by adding 0.1 mL of purified proteins. The rate of increase at A_{340} in the first 6 min was in linear range and was recorded. The rate of increase for the reaction buffer, but without a protein sample, was used as the blank. An extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used for calculating enzyme activity (Zhang *et al.*, 2006). One unit of dehydrogenase activity is defined as 1 μmol NAD^+ reduced per min. The enzyme specific-activity-calculation formula is as follows: $(A_{340} \times V) / (6.22 \times b \times W)$where A_{340} is the change in absorbance at 340 nm per min ,V the final reaction volume, b the light path, and W the amount of protein in the reaction system. A non-related protein bovine serum albumin (BSA) was used as negative control. Purification of recombinant BmADH by the Ni-NTA column increased specific dehydrogenase activity around 6-fold to 80 unit/mg (Figure 3B). This specific recombinant BmADH activity, measured by ethanol-dependent reduction of the NAD^+ reaction, was comparable to ADH activity assayed in whole-larval homogenates of *D. lebanonensis* (0.5-1 unit/mg) (Geer *et al.*, 1988).

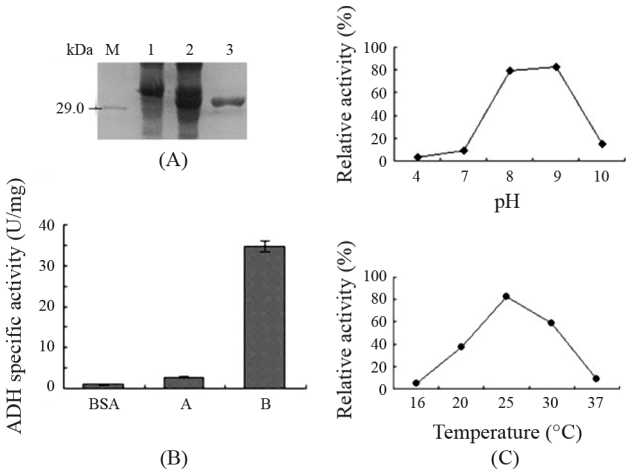

Figure 3 - Electrophoretic and enzymatic characterization and stability of BmADH. (A) SDS-PAGE stained with Commassie blue. M marker: lane 1 - lysate of host cells transformed with empty vector; lane 2 - lysate of host cells with BmADH expressionlane 3 - recombinant BmADH purified by Ni-NTA resin column. (B) Alcohol dehydrogenase specific activity, with BSA (bovine serum albumin) as negative control. A: cell lyaste of BL21 (DE3) with BmADH expression. B: purified BmADH protein. Data represent mean ± S.D. * indicates $p < 0.01$ compared to BSA. (C) Stability of alcohol dehydrogenase activity at different pHs and temperatures. Each point represents the average of duplicate determination.

Enzyme pH stability was determined by measuring enzymatic activity under standard enzyme assay conditions, at various pH levels ranging from 4.0 to 10.0. The following 0.1 M buffer systems of varying pH were used: acetate buffer (NaAc-HAc) for pH 4.0; phosphate buffer (NaH2PO4-Na2HPO4) for pH 7.0; and NaOH/glycine buffer for pHs 8.0, 9.0 and 10.0. Stability was determined by measuring specific enzymatic activity under standard enzyme assay conditions after incubating the enzyme solution for 10 min at temperatures of 16, 20, 25, 30 and 37 °C. The results obtained showed that enzyme presented high stability at pH 8.0-9.0, thermal stability being completely maintained up to 25 °C, after which activity gradually decreased (Figure 3C). In fact, many ADHs are generally unstable, low stability often hampering their industrial application (Hirakawa *et al.*, 2004)

In summary, for the first time, a *B. mori* ADH gene was identified and the recombinant BmADH enzyme experimentally characterized. Soluble recombinant BmADH proteins from *E. coli* were produced to determine substrate specificity. In the oxidoreduction reaction, BmADH catalyzed the reduction of NAD^+ to NADH in the presence of ethanol, whereby the inference that ethanol was a substrate of BmADH, and that the latter might be involved in ethanol metabolism in *B. mori*.

Acknowledgments

This work was supported by grants from the Jiangsu Sci-Tech Support Project-Agriculture (No. BE2008379), the National Program of High-tech Research and Development (863 High-Tech Program, No. 2008AA10Z145), and the National Natural Science Foundation (No. 30871826).

References

Adolph HW, Zwart P, Meijers R, Hubatsch I, Kiefer M, Lamzin V and Cedergren-Zeppezauer E (2000) Structural basis for substrate specificity differences of horse liver alcohol dehydrogenase isozymes. Biochemistry 39:12885-12897.

Atrian S, Sanchez-Pulido L, Gonzalez-Duarte R and Valencia A (1998) Shaping of *Drosophila* alcohol dehydrogenase through evolution: Relationship with enzyme functionality. J Mol Evol 47:211-221.

Benach J, Atrian S, Gonzalez-Duarte R and Ladenstein R (1999) The catalytic reaction and inhibition mechanism of *Drosophila* alcohol dehydrogenase: Observation of an enzyme-

bound NAD-ketone adduct at 1.4 A resolution by X-ray crystallography. J Mol Biol 289:335-355.

Benach J, Winberg JO, Svendsen JS, Atrian S, Gonzalez-Duarte R and Ladenstein R (2005) *Drosophila* alcohol dehydrogenase: Acetate-enzyme interactions and novel insights into the effects of electrostatics on catalysis. J Mol Biol 345:579-598.

Branden C-I, Jornvall H, Eklund H and Furugren B (1975) The Enzymes. 3rd edition. Academic Press, New York, 103 pp.

Doga A (2010) Alcohol dehydrogenase and its simple inorganic models. Coord Chem Rev 254:916-937.

Gasperi G, Kafetzopoulos D, Christodoulidou A, Bouriotis V and Savakis C (1994) Isolation and partial characterization of two alcohol dehydrogenase isozymes from the medfly *Ceratitis capitata*. Insect Biochem Mol Biol 24:87-94.

Geer BW, McKechnie SW, Bentley MM, Oakeshott JG, Quinn EM and Langevin ML (1988) Induction of alcohol dehydrogenase by ethanol in *Drosophila melanogaster*. J Nutr 118:398-407.

Hirakawa H, Kamiya N, Kawarabayashi Y and Nagamune T (2004) Properties of an alcohol dehydrogenase from the hyperthermophilic Archaeon *Aeropyrum pernix* K1. J Biosci Bioeng 97:202-206.

Huo J, Shi H, Yao Q, Chen H, Wang L and Chen K (2010) Cloning and purification of recombinant silkworm dihydrolipoamide dehydrogenase expressed in *Escherichia coli*. Protein Expr Purif 72:95-100.

Mazi V, Cosmidis N, Loukas M, Clonis Y and Zouros E (1998) Biochemical differences between products of the ADH locus in olive fruit fly (*Bactrocera oleae*). Biochem Genet 36:259-269.

Oudman L, Van Delden W, Kamping A and Bijlsma R (1991) Polymorphism at the Adh and alpha Gpdh loci in *Drosophila melanogaster*: Effects of rearing temperature on developmental rate, body weight, and some biochemical parameters. Heredity 67:103-115.

Reid MF and Fewson CA (1994) Molecular characterization of microbial alcohol dehydrogenases. Crit Rev Microbiol 20:13-56.

Rella R, Raia CA, Pensa M, Pisani FM, Gambacorta A, De Rosa M and Rossi M (1987) A novel archaebacterial NAD+-dependent alcohol dehydrogenase. Purification and properties. Eur J Biochem 167:475-479.

Williamson VM and Paquin CE (1987) Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol Gen Genet 209:374-381.

Zhang ZH, Kang YM, Yu Y, Wei SG, Schmidt TJ, Johnson AK and Felder RB (2006) 11beta-hydroxysteroid dehydrogenase type 2 activity in hypothalamic paraventricular nucleus modulates sympathetic excitation. Hypertension 48:127-133.

Associate Editor: Carlos F.M. Menck