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

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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 PCR, ADH, 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 presumably 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., 1999), *Drosophila lebanonensis* (Benach et al., 1999), *Ceratitis capitata* (Mediterranean fruit flies) (Gasperi et al., 1994) and *Bactrocera (Dacus) oleae* (olive fly) (Mazı 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. rswwd0_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

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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 (DIADH) and *D. melagaster* 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).

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 OD₆₀₀ 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).
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 A340 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: (A340 x V) / (6.22 x b x W) where A340 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).

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).

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