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# Cloning and purification of recombinant silkworm dihydrolipoamide dehydrogenase expressed in *Escherichia coli*

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ABSTRACT

Dihydrolipoamide dehydrogenase (DLDH), a flavin-dependent oxidoreductase is essential for energy metabolism. As an oxidoreductase it catalyzes the $NAD^+$-dependent oxidation of dihydrolipoamide. In this study, a putative *Bombyx mori* dihydrolipoamide dehydrogenase (BmDLDH) gene was cloned, expressed, purified and characterized for the first time. The BmDLDH gene was amplified from a pool of silkworm cDNAs by PCR and cloned into *Escherichia coli* expression vector pET-28a(+). The recombinant His-tagged BmDLDH protein was expressed in *E. coli* BL21 (DE3) and purified by metal chelating affinity chromatography. The amino acid sequence of recombinant protein was confirmed by mass spectroscopic analysis. Furthermore, the oxidoreductase activity in the reverse reaction indicated that the soluble recombinant BmDLDH produced at lower growth temperature was able to catalyze the lipoamide-dependent oxidation of NADH.



## Introduction

Dihydrolipoamide dehydrogenase (EC 1.8.1.4), a member of the oxidoreductase family, is a component of pyruvate dehydrogenase complex (PDHC), α-keto-glutarate dehydrogenase complex (KGDC), and branched-chain α-keto acid dehydrogenase complex (BCKDC) [1–3]. DLDH[1] is also a component of the glycine cleavage system [4], which participates in glycine metabolism in eukaryotes. The DLDH protein has four distinctive subdomain structures: FAD-binding, $NAD^+$-binding, central, and interface domains and usually forms homodimer in mitochondria [5]. DLDH catalyzes the $NAD^+$-dependent oxidation of dihydrolipoamide: dihydrolipoamide + $NAD^+$ ↔ Lipoamide + NADH + $H^+$ [6]. DLDH also has diaphorase activity, being able to catalyze the oxidation of NADH to $NAD^+$ by using different electron acceptor such as $O_2$ [7], labile ferric iron [8], nitric oxide [9], and ubiquinone [10,11]. In addition, DLDHs from human, mouse and pig can function as a moonlighting protease in cleavage of mitochondrial iron chaperone frataxin [12]. Recently, DLDH from mesophilic fungi *Starkeyomyces koorchalomoides* is characterized as an acetyltransferase [13].

As the basis of versatile functions of DLDH is far from being elucidated, pure and functional DLDH of different species is needed for investigations. Previously, human DLDH and mutants were expressed in *Escherichia coli* for functional and structural studies [12,14–18]. DLDHs of soybean, *Mycobacterium tuberculosis* and *Haloferax volcanii* were also expressed and purified from *E. coli* for characterization of the enzyme [19–21]. However, DLDHs from insects have not been well characterized. Since insects represent the most abundant and diverse group of organisms in any phylum, their number and diversity far exceeding species in all other phyla combined [22], they are interesting candidates to study evolutionary, genetic and biochemical functions of multi-enzyme complexes. As the domesticated silkworm *B. mori* is a model insect, study of BmDLDH will help to elucidate the functional role of insect DLDH. In this present work, the open reading frame (ORF) of BmDLDH was cloned and the recombinant enzyme was expressed in *E. coli*. The amino acid sequence of recombinant BmDLDH was verified by mass spectroscopic analysis. The oxidoreductase activity of the recombinant BmDLDH protein was assayed.

## Materials and methods

### Materials

The expression vector pET-28a(+) and *E. coli* strain BL21 (DE3) were obtained from Novagen (CA, USA). Oligo-dT primers, RNase-free DNaseI, Taq polymerase, restriction enzymes, T4 DNA ligase, DNA markers, protein markers and the subcloning vector

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[1] *Abbreviations used:* DEPC, diethylpyrocarbonate; DLDH, dihydrolipoamide dehydrogenase; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; $NAD^+$, nicotinamide adenine dinucleotide; PVDF, polyvinylidene difluoride; PBS, phosphate buffered saline; PBST, phosphate buffered saline with 0.1% tween-20; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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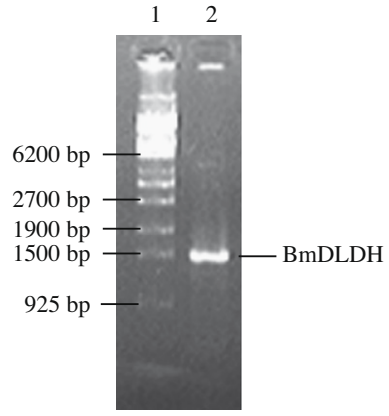


**Fig. 1.** Agarose gel electrophoresis of PCR products. Lane 1: DNA marker; lane 2: PCR product amplified from *B. mori* cDNAs with BmDLDH specific primers.

pMD18-T were purchased from TaKaRa (Dalian, China). Chemicals are all from Sigma (MO, USA) or a domestic provider in China if not stated otherwise. *B. mori* strain C108 was reared in our lab.

*RNA extraction and cDNA synthesis*

The fifth instar larvae of *B. mori* were dissected, and washed with cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$ and 1.4 mM $KH_2PO_4$). Then the tissue sample was frozen immediately in liquid nitrogen and stored at −80 °C until extraction of RNA. Total RNA was extracted from frozen sample with RNeasy® Mini Kit (Qiagen). DNA was digested with RNase-free DNaseI at 37 °C for 20 min. The RNA was further extracted with phenol–chloroform and precipitated with ethanol. The RNAs dissolved in DEPC-treated $ddH_2O$ were used to make cDNAs with the SuperScript II™ reverse transcriptase (Promega) and oligo-dT primers following the manufacturer's instructions.

*Construction of expression vector containing BmDLDH gene*

The BmDLDH specific primers, forward primer (5′AGGATCCAT GGGCTATAAATTTCTAAA3′) with a BamHI site (underlined), and reverse primer (5′ACTCGAGTTAGAAGTTGATTGGTTTTC3′) with an XhoI site (underlined) were designed to amplify the ORF of a putative BmDLDH gene (GeneBank accession number AF529135). The PCR reaction was carried out with 35 amplification cycles (94 °C for 60 s, 52 °C for 45 s, and 72 °C for 90 s) in a Gene Amp 2400 System thermocycler. The PCR product was ligated into pMD18-T vector using T4 DNA ligase and then transformed into *E. coli* TG1. A fragment between BamHI and XhoI containing the BmDLDH gene was excised from the recombinant plasmid. The purified fragment was subcloned into the pET-28a(+) expression vector and transformed into *E. coli* BL21 (DE3). DNA sequencing confirmed that the BmDLDH ORF was correctly fused to the N-terminal 6 × His-tag.

*Expression and purification of recombinant protein*

To express recombinant protein, a freshly transformed colony was cultured in LB medium supplement with kanamycin (50 μg/ml) at 37 °C overnight. This overnight culture was inoculated into fresh LB medium and cultured at 37 °C with vigorous shaking. When $OD_{600}$ reached 0.4, the expression of BmDLDH was induced with IPTG (final concentration 0.1–2 mM during optimization) and further cultured at 16 °C for 20 h. Cells were harvest by centrifugation (4500*g*, 4 °C, 15 min) and SDS–PAGE analysis on a 10% gel was performed to estimate the expression level of BmDLDH. 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), then the cell suspension was lysed by sonication. The lysate was clarified by centrifugation (16,000*g*, 4 °C, 25 min). The supernatant was loaded onto a Ni-NTA affinity column (Qiagen). Purification conditions

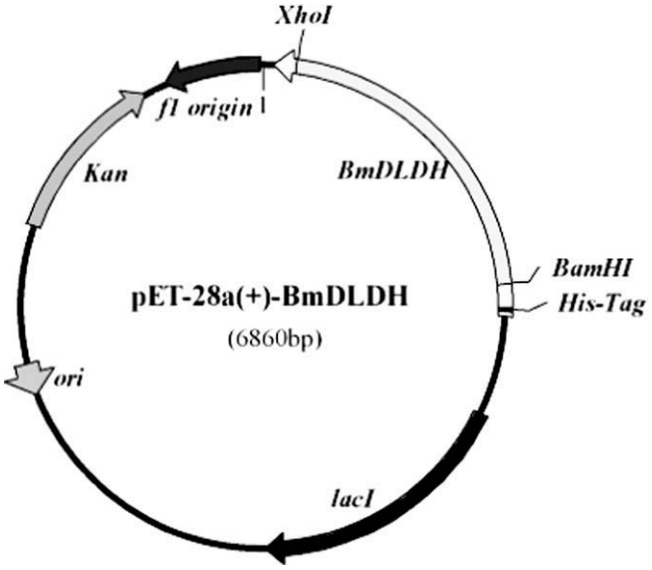


**Fig. 3.** Map of BmDLDH expression plasmid. The open reading frame of BmDLDH was inserted into expression vector pET-28a(+) with BamHI and XhoI.

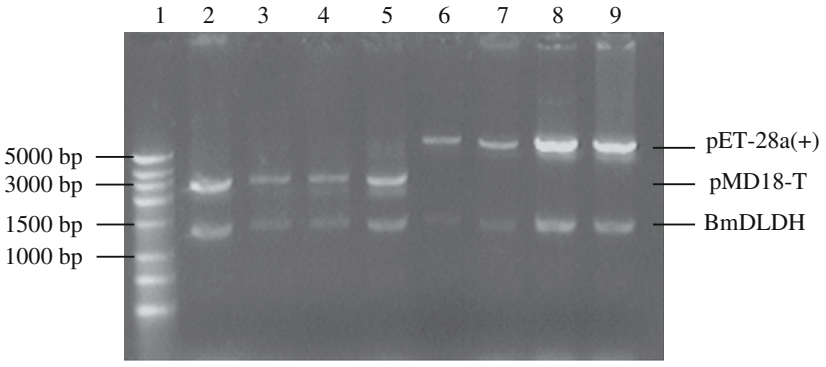


**Fig. 2.** Agarose gel electrophoresis of recombinant plasmids digested with BamHI and XhoI. Lane 1: DNA marker; lanes 2–5: pMD18-T-BmDLDH from four different colonies; lanes 6–9: pET-28a(+)-BmDLDH from four different colonies.