Protein Expression and Purification 137 (2017) 20–25

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

White shrimp *Litopenaeus vannamei* recombinant lactate dehydrogenase: Biochemical and kinetic characterization

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ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form
14 June 2017
Accepted 14 June 2017
Available online 15 June 2017

Keywords:
LDH
Biochemical and kinetic characterization
Shrimp
Enzyme kinetics
Homotetramer

ABSTRACT

Shrimp lactate dehydrogenase (LDH) is induced in response to environmental hypoxia. Two protein subunits deduced from different transcripts of the LDH gene from the shrimp *Litopenaeus vannamei* (LDHvan-1 and LDHvan-2) were identified. These subunits are expressed by alternative splicing. Since both subunits are expressed in most tissues, the purification of the enzyme from the shrimp will likely produce hetero LDH containing both subunits. Therefore, the aim of this study was to overexpress, purify and characterize only one subunit as a recombinant protein, the LDHvan-2. For this, the cDNA from muscle was cloned and overexpressed in *E. coli* as a fusion protein containing an intein and a chitin binding protein domain (CBD). The recombinant protein was purified by chitin affinity chromatography column that retained the CBD and released solely the full and active LDH. The active protein appears to be a tetramer with molecular mass of approximately 140 kDa and can use pyruvate or lactate as substrates, but has higher specific activity with pyruvate. The enzyme is stable between pH 7.0 to 8.5, and between 20 and 50 °C with an optimal temperature of 50 °C. Two pK_a of 9.3 and 6.6, and activation energy of 44.8 kJ/mol°K were found. The kinetic constants K_m for NADH was 23.4 ± 1.8 μM, and for pyruvate was 203 ± 25 μM, while V_{max} was 7.45 μmol/min/mg protein. The shrimp LDH that is mainly expressed in shrimp muscle preferentially converts pyruvate to lactate and is an important enzyme for the response to hypoxia.

1. Introduction

Shrimp farming is a very important economic activity in many countries. Mexico is one of the largest producers of the white shrimp *Litopenaeus vannamei* in the world, one of the most cultivated species. However, environmental factors such as temperature, salinity and changes in the concentration of oxygen may affect this production. Several crustaceans in response to low oxygen environments -hypoxia- migrate to avoid hypoxic areas and hyperventilate to increase oxygen supply to the cells. If hypoxia persists, other physiological, biochemical and molecular mechanisms are activated, including anaerobic glycolysis. In the shrimp *L. vannamei*, accumulation of lactate occurs during moderate hypoxia with 2–2.6 mg of dissolved oxygen per liter (DO) for a few weeks [1], and also, during severe hypoxia of 0.4 mg DO [2]. Lactate dehydrogenase -LDH (EC 1.1.1.27)- is the terminal enzyme in anaerobic glycolysis in many organisms and catalyzes the interconversion of lactate and pyruvate using NAD as coenzyme [3].

The LDH from human and other vertebrates have been exhaustively studied for many decades, but there are few full reports for LDH from crustaceans. As in vertebrates, invertebrate organisms contain multiple forms of LDH [4]. The first report of multiple forms of LDH in invertebrates came from the studies in the lobster *Homarus americanus* [5]. But also, some crustaceans as the snow crab *Chionoecetes opilio* [6], the crayfishes *Orconectes limosus* [7] and *Procambarus clarki* [8], the Northern and Antarctic krills *Meganyctiphanes norvegica* and *Euphausia superba* [3] and the

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http://dx.doi.org/10.1016/j.pep.2017.06.010
1046-5928/

isopod *Saduria entomon* [9], to name a few, all have isoforms of LDH that are mainly tetramers of approximately 130–160 kDa, and are composed of one or two subunits types and, as in vertebrate enzymes, have tissue-specificity [3]. The crustacean LDH isoenzymes that predominate are represented by five isoforms generated by the expression of two independent loci (*ldh-A* and *ldh-B*) [3,5,6,9]. The LDH-A4 isoenzyme is found predominantly in anaerobic tissues as muscle (M-type LDH), whereas the LDH-B4 isoform predominates in aerobic tissues as heart (H-type LDH). In the penaeid shrimp *L. vannamei*, two subunits of LDH that are derived from a unique gene by mutually exclusive alternative splicing were predicted from the cDNA nucleotide sequence and shown to be differently expressed in response to hypoxia. One of the subunits predominates in gill and is called LDHvan-1; the second subunit predominates in muscle and is called LDHvan-2. These two differ in the inclusion of exon 5 in LDHvan-1 or exon 6 in LDHvan-2 and have a 95% identity at the amino acid level. Interestingly, LDH activity also changes in response to hypoxia [10]. To study the biochemical and kinetics characteristics of LDH from *L. vannamei*, the coding sequence of one of the protein subunit LDHvan-2, from herein on called LDH-2, was cloned and the recombinant protein overexpressed in *E. coli* and purified.

2. Materials and methods

2.1. Cloning and overexpression of recombinant LDH-2

The Intein Mediated Purification with Affinity Chitin-binding Tag IMPACT system (New England Biolabs) was used for cloning, overexpression and purification of the recombinant LDH-2 with some modifications. The coding sequence was obtained from the recombinant plasmid containing the complete cDNA previously reported [10] by PCR amplification with the primers LDHNheIFw (AATAATGCTAGC*ATGGCCTCTGTTCCTG*) and LDHXhoIRv (AGTAATCTCGAG*GAACTGAATTCCGGCC*). These primers contained the restriction sites for *NheI* in the 5'end preceding the initial MET and a *XhoI* in the 3′ end, omitting the stop codon. Also, six bases more were added to each primer before the restriction sites to facilitate digestion with the restriction enzymes; the coding sequences are in italics. The PCR product and the pTXB1 plasmid vector were digested with the restriction enzymes, ligated and used to transform TOP10 *E. coli* chemically competent cells. Recombinant positive clones were sequenced to assure that the correct sequence was obtained to produce the fusion protein. This construct produced a fusion protein that added in the carboxyl terminal of the LDH an intein with a chitin protein binding domain. The *E. coli* strain ER2566 was used to produce the recombinant protein following the recommendations of the IMPACT system. Basically, the recombinant clone was grown in 1 l of LB medium containing 125 μg/mL of ampicillin. Expression of the protein was induced by adding 0.2 mM IPTG when the culture reached an optical density of 0.6 at 600 nm and incubated for 2.5 h at 37 °C. The bacterial pellet was collected by centrifugation at 5000×*g* at 4 °C for 15 min and stored at −80 °C until used. Aliquots of the samples were collected throughout the procedure and analyzed in a 13% SDS-PAGE in reducing conditions according to Laemmli [11]. The gels were stained with colloidal Coomassie blue G-250 (10% ammonium sulfate, 0.1% Coomassie G-250, 3% orthophosphoric acid, 20% ethanol and HPLC water) [12,13].

2.2. Purification of recombinant LDH-2

The bacterial pellets were resuspended in 10% volume of the original culture in column buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.5), plus 20 μM phenylmethylsulfonyl fluoride (PMSF) and 0.25% Triton X-100, sonicated with 4 pulses of 10 s each interrupted by pauses of 10 s, while maintaining the solution on ice, followed by centrifugation at 15,000×*g* for 30 min at 8 °C. The affinity column containing 7 mL of immobilized chitin was washed with 10 vol of column buffer (70 mL) before loading the sample and the sample was recirculated once. The column was washed with 20 vol (140 mL) of column buffer to remove the unbound proteins. To promote intein cleavage, 21 mL of cleavage buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 50 mM DTT, pH 8.5) were loaded to the column at 1 mL/min and then, incubated for 19 h at 21 °C. Finally, the LDH-2 was eluted using column buffer. Samples from each purification step were analyzed in 13% reducing SDS-PAGE and stained with colloidal Coomassie blue G-250 as mentioned before or with silver. Silver staining was done by incubating the gel in 30% ethanol, 10% acetic acid for 30 min, then in 30% ethanol, 4.1% sodium acetate, 0.13% sodium thiosulfate, 0.52% glutaraldehyde for 30 min and washed with deionized water for 5 min with gentle shaking three times. Subsequently, the gel was incubated with 20% ethanol, 0.1% silver nitrate, 0.02% formaldehyde for 20 min, followed by 20% ethanol, 2.5% sodium carbonate, 0.01% formaldehyde for developing and the reaction was stopped by washing the gel with 1.46% EDTA [14].

2.3. Protein quantification

The recombinant protein was quantified by the method of Bradford [15] adapted to a 96 well microplate and done in triplicate. For this, 20 μL of the sample was mixed with 200 μL of Bradford reagent (0.01% Coomassie blue G-250, 4.7% ethanol, 8.5% H_3PO_4), incubated for 5 min at room temperature and the absorbance at 595 nm was recorded. A standard curve of bovine serum albumin was used to calculate the protein concentration in the samples.

2.4. Zymogram for recombinant LDH-2

Native 8% polyacrylamide gels were used to detect LDH activity using lactate as substrate [16]. An aliquot of 15 μg of pure LDH-2 was loaded onto the gel and run at 15 mA, at 4 °C. The gel was incubated at 37 °C for 1.5 h in 140 mM Tris-HCl, pH 8; 70 mM L-lactate, pH 8; NAD^+ (0.3 mg/mL); NBT (0.08 mg/mL); phenazine methosulfate (0.08 mg/mL) until dark blue bands appeared. Native proteins marker (HMW Native Marker kit, GE Healthcare Life Science) that contains bovine heart LDH was used to estimate the molecular weight of the active recombinant LDH-2 and as a positive control for the detection of enzyme activity.

2.5. Spectrophotometric determinations

The enzymatic activity of the recombinant LDH-2 was determined by spectrophotometry, as described by Worthington (standard method) [17] using pyruvate with the following modifications. Oxidation of NADH was measured by the decrease in absorbance at 340 nm for 5 min at 25 °C. The reaction mixture contained 100 mM Tris-HCl, pH 7.6; 4 mM pyruvate; 0.33 mM NADH, and the reaction was initiated by adding 50 μL of the purified enzyme (0.02 mg/mL); the final volume of the reaction was 0.5 mL. Each determination was done in triplicate and the specific activity is expressed as μmoles of NAD^+ produced/min/mg protein [17].

2.6. Optimum pH and stability assays

The stability to pH was determined at 25 °C by incubating the enzyme for 1 h in the following buffers: 100 mM MES (pH 5.5–6.5), 100 mM HEPES (pH 6.5–8.0), 100 mM Tris-HCl (pH 7.0–9.0), 100 mM CHES (pH 8.5–10) and 100 mM Bis-Tris propane (pH