

## Research Article

# Expression of the Lactate Dehydrogenase Gene from *Eptatretus okinoseanus* in *Escherichia coli*

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Amplified *Eptatretus okinoseanus* cDNA was digested with NdeI and EcoRI, cloned into pCold trigger factor (TF), and transformed with *Escherichia coli* strain BL 21 in which a csp A promoter was introduced to inhibit the expression of foreign peptides. Recombinant lactate dehydrogenase (LDH) was obtained in the soluble fraction after sonication of the cells. The protein was digested by HRC 3C protease, thrombin, and factor Xa. The specific activity of TF-tagged protein and tagless protein were  $0.646 \times 10^6$  mIU/mg and  $3.56 \times 10^6$  mIU/mg, respectively. The deletion of the TF tag enhanced the activity compared with the native protein to  $13.4 \times 10^6$  mIU/mg, showing that this expression method is effective for the mass production of the protein to allow further study of the structure of LDH.

## 1. Introduction

Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27; LDH) is a key enzyme in the glycolytic reaction cascade due to its metabolic significance in catalyzing the terminating step. The physicochemical properties of the enzyme have been described in detail [1–5]. In most vertebrates, the LDH molecule forms a tetrameric structure [6] and two separate loci that encode the A and B subunits constructing five tetrameric isozymes have been identified [7]. There has been great interest in LDH isozymes since their occurrence varies during evolution [8], fetal development [9], and cancer development [10]. We previously reported the enzymatic properties of LDHs of several hagfish species, which are prototype fish living at a wide range of depths [11–15]. The skeletal muscle of two hagfish, *Eptatretus okinoseanus* and *Paramyxine atami*, and of a lamprey, *Entosphenus japonicus*, has the A<sub>4</sub> isozyme whereas the B<sub>4</sub> isozyme was expressed in the heart of the hagfish *E. okinoseanus* and *P. atami*, but not in *E. japonicus*. No heteroisozymes were detected in Cyclostomata [13]. In 2004, Johns and Somero [16] proposed the heat-tolerant structure of Pomacentridae LDH,

and in 2008 Brindley et al. [17] reported the pressure-tolerant structure of Gadidae LDH-B and proposed 21 amino acid residues responsible for it. Recently, we have sequenced LDH-A genes from three hagfish species [13], examined their activities under high pressure, proposed seven amino acid residues responsible for their pressure tolerance [15, 18], and found that the dissociation of tetramers caused the inactivation of *Eptatretus burgeri* LDH under high pressure [19]. This study aimed to determine a method for the effective expression of the LDH gene in *Escherichia coli* to obtain sufficient proteins to investigate the pressure-resistant structure using X-ray analysis.

Many studies on the expression of LDH in *E. coli* have been reported [20, 21], but almost all were on fusion proteins or proteins with relatively large amino acid residues with properties different from the native one in their three-dimensional structures and enzyme activities. In this study, we examined the optimum conditions to remove the tag of fused proteins. We are currently performing site-directed mutagenesis of the predicted amino acids and will express those mutagenic LDHs in *E. coli* using this method to examine the mechanism of pressure tolerance.

## 2. Materials and Methods

*Eptatretus okinoseanus* was collected from Suruga Bay off Shizuoka, Japan. The sampling location was 34-35° N, 138-139° E at depths of approximately 700–1000 m (ambient seawater temperature 5–8°C). Skeletal muscles were washed with phosphate-buffered saline (PBS; 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl) pretreated with diethylpyrocarbonate to remove RNase and immediately frozen at –80°C.

Messenger RNAs (mRNAs) were isolated from homogenates of skeletal muscle following the method of Loening [22]. Total cDNA was subjected to PCR with the following two primers: F1, 5'-TCGAAGGTAGGCATATGTGACCG-TGCGGGAACGT-3'; R1, 5'-CGACAAGCTTGAATTCTT-ACAGTTTCAGGTCCTTC-3'. A typical thermal cycling profile consisted of 30 cycles of the following three steps: denaturation at 98°C for 10 s; annealing at 55°C for 10 s; extension at 72°C for 45 s. The cycling steps were preceded by 2-minute denaturation at 94°C followed by 7-minute extension at 72°C. The amplified cDNA was digested with NdeI and EcoRI and cloned into pCold trigger factor (TF; Takara Bio Inc., Tokyo, Japan) into which a csp A promoter, active only at low temperature, was inserted. The *E. okinoseanus* LDH-A open reading frame (ORF) was inserted asymmetrically into the NdeI and EcoRI sites of the pCold TF vector. The sequence of the resulting plasmid was confirmed using an Applied Biosystems 3730 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

The plasmid was transformed with *E. coli* strain BL 21. Luria-Bertani broth with 100 µg/mL ampicillin was inoculated into the overnight culture of the transformed *E. coli*. When the absorbance at 600 nm reached a value of 0.5, β-D-thiogalactopyranoside was added at a final concentration of 1 mM, and the cultures were allowed to stand at 15°C for 24 h. The bacteria were harvested by centrifugation at 5,000 × g for 15 min. Cell pellets were resuspended and disrupted using a sonicator in PBS. After centrifugation at 12,000 × g for 20 min, the supernatant was applied to a cobalt-based immobilized method affinity chromatography resin column (IMAC, Clontech Laboratories Inc., Mountain View, CA, USA). After washing the column with 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8.0) containing 20 mM imidazole, tagged LDH was eluted with 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 8.0) containing 300 mM imidazole. The purified tagged LDH was confirmed with SDS-PAGE. The LDH tagged with the TF, a 48-kDa protein, was cleaved with three restriction enzymes, HRC 3C protease, thrombin, and factor Xa (Figure 1). The cleaved samples were subjected to electrophoresis using polyacrylamide gel. After electrophoresis, the gel was stained with EzStain Reverse (ATTO Corporation Tokyo, Japan), which did not inhibit LDH activity in contrast to Coomassie Brilliant Blue. The bands of LDH were cut from the gel, and LDH was extracted from the gel using ATTOPREP MF (ATTO Corporation).

LDH activity was determined in an assay medium (0.83 mM pyruvate, 0.13 mM NADH, 0.1 M phosphate buffer, pH 6.2) at 30°C. One unit of activity was defined as 1 µmol

of product (NAD<sup>+</sup>) formed from the reduction of pyruvate to lactate per minute per milligram of protein.

## 3. Results and Discussion

Figure 1 shows the genetic engineering scheme for the construction of the *E. okinoseanus* LDH-A ORF and pCold TF vector. The recombinant DNA has the csp A promoter to upregulate the target protein production at lower incubation temperatures (15°C). At 15°C, the expression of other cellular proteins is inhibited. The TF causes the expressed protein to become soluble in *E. coli*. The disruption of the *E. coli* cell wall to liberate active protein was attempted using two methods. One was disruption with zwitterionic detergent (BugBuster Protein Extraction Reagent; Takara Bio Inc., Tokyo, Japan). In this treatment, all LDH activity was lost. The other method was sonication (100 W, 10 min), after which LDH activity remained.

Figure 2 shows the results of SDS-PAGE of the soluble fraction (lane S), insoluble fraction (lane I), and tagged LDH in the soluble fraction purified with IMAC (lane P). Almost all of the expression of recombinant protein occurred in the soluble fraction, with insignificant expression in the insoluble fraction. There were only small amounts of foreign proteins (lane T), showing that csp A effectively inhibited the expression of the original *E. coli* proteins. Since the TF tag decreases the activity of recombinant LDH, we attempted to remove the tag. Column chromatography resulted in significant purification of the recombinant LDH. Figure 1 shows the cleavage sites of the three proteases. HRV 3C protease retained 17 amino acid residues, thrombin retained eight, and factor Xa retained one, indicating that the third protease was the optimal treatment to obtain nearly natural LDH. Figure 3 shows the digestion of tagged LDH by the three proteases. LDH was treated with 0.1–5 U of HRV 3C protease at 4°C for 16 h, and it was found that 5 U was the optimal dose (lanes 2–4). The results of treatment with thrombin at 25°C for 16 h showed that 5 U was optimal (lanes 5–7). After digestion by factor Xa 0.1–5 U at 25°C for 16 h, no clear peptide bands were observed (lanes 8–10), and the reason for this is illustrated in Figure 4. Isolation of the LDH cleaved by HRV 3C protease was performed using IMAC. LDH passed through the column, although approximately 20% of the TF tag also passed through the column and thus purification was not achieved. Thrombin-digested LDH was also not purified, since thrombin passed through the column with LDH (data not shown). Digestion of tagged LDH by factor Xa was performed using a modified method to avoid salting-out under the cleavage conditions shown in Figure 3. The reaction mixture was diluted 10-fold with distilled water. Figure 4 shows the results of digestion by 0.1–5 U of factor Xa in the modified method at 20°C for 16 h (lanes 2–4). A band of digested LDH was identified after treatment with 1 U of factor Xa (lane 3). Factor Xa-digested LDH was subjected to electrophoresis with gradient PAGE and stained with Coomassie Brilliant Blue (Figure 5). Those bands were broad since SDS was absent. Recombinant LDH (lane 2) was isolated around the same migrating



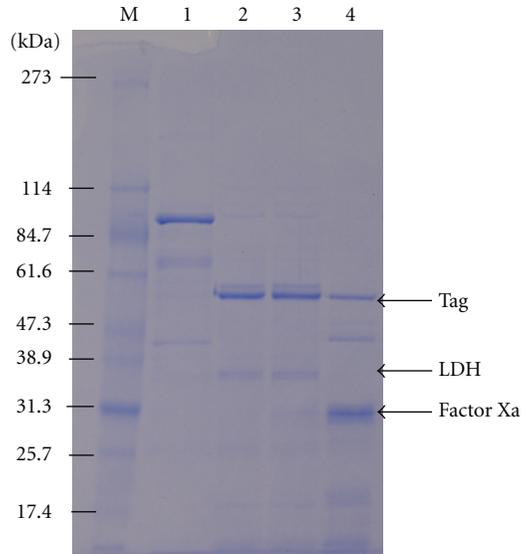


FIGURE 4: Digestion by factor Xa using the modified method. M: molecular size markers, 1: TF-tagged LDH, 2: digestion by 0.1 U, 3: digestion by 1 U, 4: digestion by 5 U. To avoid salting-out under the cleavage conditions shown in Figure 3, the reaction solution was diluted by 10-fold with distilled water.

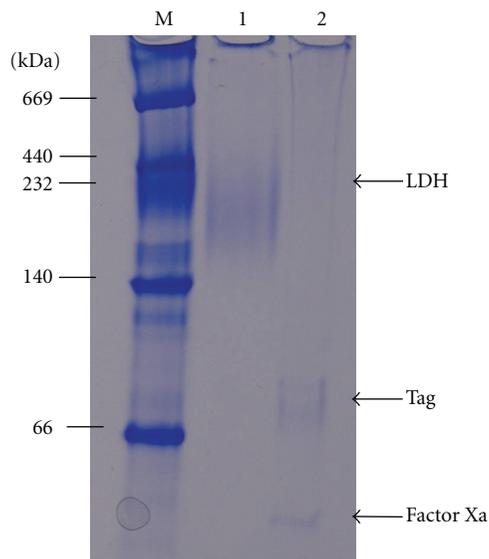


FIGURE 5: Gradient (5–20%) polyacrylamide gel electrophoresis. The tagged LDH was digested by factor Xa and subjected to electrophoresis. M: molecular size markers, 1: LDH purified from muscle, 2: fusion protein digested by 1 U of factor Xa.

is the first report comparing the activities of expressed LDH with and without a tag and those of the native enzyme.

We are currently performing site-directed mutagenesis of LDH and expressing it in *E. coli*. The mechanism of adaptation of LDHs from deep-sea hagfish to high pressure will be clarified at the amino acid sequence level in our ongoing experiments.

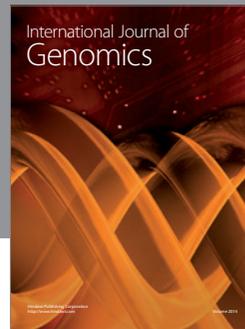
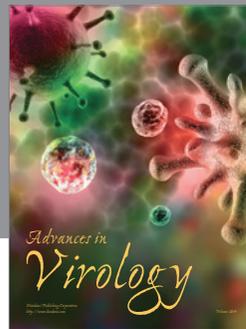
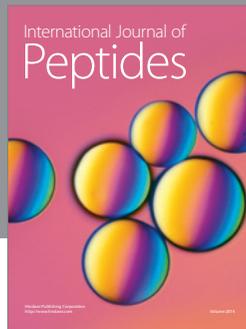
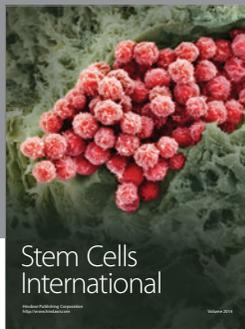
TABLE 1: Specific activities of purified LDH from muscle, tagged LDH, and tag-less LDH.

	Specific activity ( $\times 10^6$ m IU/mg)
Purified LDH from muscle	13.4
LDH expressed in <i>E. coli</i>	
with TF-tag	0.646
without TF-tag	3.56

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