Cloning, expression, purification, crystallization and X-ray crystallographic analysis of D-lactate dehydrogenase from *Lactobacillus jensenii*

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The thermostable D-lactate dehydrogenase from *Lactobacillus jensenii* (LjD-LDH) is a key enzyme for the production of the D-form of lactic acid from pyruvate concomitant with the oxidation of NADH to NAD⁺. The polymers of lactic acid are used as biodegradable bioplastics. The LjD-LDH protein was crystallized using the hanging-drop vapour-diffusion method in the presence of 28% (w/v) polyethylene glycol 400, 100 mM Tris-HCl pH 9, 200 mM magnesium sulfate at 295 K. X-ray diffraction data were collected to a maximum resolution of 2.1 Å. The crystal belonged to space group *P*3₁21, with unit-cell parameters *a* = *b* = 90.5, *c* = 157.8 Å. With two molecules per asymmetric unit, the crystal volume per unit protein weight (*V*_M) is 2.58 Å³ Da^{−1}, which corresponds to a solvent content of approximately 52.3%. The structure was solved by single-wavelength anomalous dispersion using a selenomethionine derivative.

1. Introduction

The thermostable D-lactate dehydrogenase (D-LDH) from *Lactobacillus jensenii* is a key enzyme for the production of lactic acid from pyruvate concomitant with the oxidation of NADH to NAD⁺. Lactic acid has a single chiral carbon. Lactate dehydrogenases exist in four distinct enzyme classes. Each one acts on either D-lactic acid (produced by D-lactate dehydrogenase; D-LDH) or L-lactic acid (produced by L-lactate dehydrogenase; L-LDH) (Datta *et al.*, 1995). The classes are cytochrome-*c*-dependent and NAD-dependent enzymes. L-LDHs have been intensively studied because their product (L-lactate) is widely used in food, cosmetics and medical applications (Liu, 2003; Singh *et al.*, 2006). In contrast, little is known about D-LDHs (Narins *et al.*, 2010; Richter *et al.*, 2011). Recent reports have demonstrated that D-LDH belongs to a family of NAD-dependent enzymes and has a completely different fold from L-LDH (Kochhar *et al.*, 2000; Razeto *et al.*, 2002).

Polylactic acid (PLA) is a biodegradable polyester polymer of lactic acid which is mainly produced through a bacterial fermentation process. The PLA copolymer, consisting of a mixture of the enantiomers poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA), can potentially act as a high-performance polymer as a result of its increased melting point and improved mechanical properties compared with racemic PLA (Lunt, 1998; de Jong *et al.*, 2001; Tsuji, 2002). The efficient production of optically pure D-lactic acid is required for the preparation of PDLA on a technical scale. The most well known lactic acid producing organisms, such as *Lactobacillus* and *Lactococcus* species, are members of the taxonomic order Lactobacillales and are also commonly referred to as lactic acid bacteria. Lactobacilli generally have high productivity, but have special and often expensive nutrient requirements. Not all microorganisms produce stereochemically pure lactic acid. The synthesis of enantiopure D-lactic acid by bacterial fermentation on an industrial scale is far more difficult compared with that of L-lactic acid. Several natural D-lactic acid producing bacterial species exist, such as *Sporolactobacillus inulinus*, *S. laevolacticus* and *Lactobacillus delbrueckii*

Table 1
Ljd-LDH production information.

Source organism	<i>L. jensenii</i>
DNA source	Chromosomal DNA
Forward primer	5'-GCGGCGATATGAAATCGGTCTTCTCC-3'
Reverse primer	5'-GCGCGAATTCGGGCGGACACAAATGC-3'
Cloning vector	pET-22b(+)
Expression vector	pET-22b(+): <i>Ljd</i> -LDH
Expression host	<i>E. coli</i> B834
Complete amino-acid sequence of the construct produced	MKIAVFSPESEKRLVAATEKKFGCELKLDSELSAENVQV- ADCDGVLLKPLGNLDEIVYKKLADYGIKSLRIVGTNT- IDFDLAKKYHLTVTNVVSPIRAIAEMAVTQAMYLNRKIG- EFKANMDKGFNTNPSLISNEIYNKTIGLVGHIGSAVA- QIFSAMGAKVLAYDVIYNPEVEPYLYADFDTLKEADII- SLHTPLLKSTENMIGKKQFAEMKNDAILNAARGELVDTA- ALIEALEKHEIAAAGLDTLAHESSYFFKKVDDAQIPADYK- KLAAMPNVIVTPHSAYFTKTSVRNMIEISLRDTIALANGE- RAHFVVSRRNSSVDKLAALAEHHHHH

(Lamzin *et al.*, 1994; Dengler *et al.*, 1997). Therefore, the discovery or engineering of highly active and thermostable D-LDHs is a prerequisite for the economical production of D-lactic acid. As a step towards elucidating the structural and biochemical properties of a thermostable D-LDH from *L. jensenii* (*Ljd*-LDH), the closest structurally known homologue of which is the D-isomer-specific 2-hydroxyacid dehydrogenase from *L. bulgaricus*, with an amino-acid sequence identity of 68% (Jun *et al.*, 2012), we cloned the *Ljd*-LDH coding gene and purified the recombinant *Ljd*-LDH protein. Crystals of *Ljd*-LDH of diffraction quality were obtained by the hanging-drop method.

2. Expression and purification of the recombinant *Ljd*-LDH

Forward and reverse primers were designed (Table 1) to introduce *Nde*I and *Eco*RI cleavage sites, respectively. The *Ljd*-LDH coding gene was amplified from the *L. jensenii* chromosome by polymerase chain reaction (PCR). The PCR product was then subcloned into pET-22b(+) (Merck Millipore), resulting in a 6×His tag at the C-terminus. The resulting expression vector pET-22b(+):*Ljd*-LDH was transformed into *Escherichia coli* B834 strain and the cells were grown overnight in Luria Broth (LB) medium containing 100 µM ampicillin at 310 K. At an optical density at 600 nm (OD_{600}) of 0.6,

Ljd-LDH protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h at 291 K, the cells were harvested by centrifugation at 4000g for 15 min at 277 K.

The cell pellet was resuspended in buffer A (40 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13 500g for 15 min and the lysate was applied onto a Ni-NTA agarose column (Qiagen). After washing with buffer A containing 20 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. Finally, the trace amount of contaminants was removed by size-exclusion chromatography using a Superdex 200 prep-grade column (320 ml, GE Healthcare) equilibrated with buffer A containing 1 mM dithiothreitol (DTT). The protein eluted at a molecular weight of 37.5 kDa, which corresponds to the calculated molecular weight of the *Ljd*-LDH monomer (Fig. 1). All purification experiments were performed at 277 K. The purified protein was concentrated to 35 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT. The selenomethionine (SeMet) derivative of the *Ljd*-LDH protein was expressed in M9 minimal medium (Maniatis *et al.*, 1982) supplemented with 25 mg l⁻¹ SeMet and was purified in the same manner as the wild type. Macromolecule-production information is given in Table 1.

3. Crystallization

Crystallization of the purified *Ljd*-LDH protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald Bio using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 1.5 µl protein solution (35 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT) with 1.5 µl reservoir solution and then equilibrating it against 0.5 ml reservoir solution. *Ljd*-LDH crystals were observed from several crystallization screening conditions. After several steps of improvement using the hanging-drop vapour-diffusion method, crystals of the best quality appeared in one week and reached maximum dimensions of 0.25 × 0.1 × 0.1 mm using 28% (w/v) polyethylene glycol (PEG) 400, 100 mM Tris-HCl pH 9.0, 200 mM magnesium sulfate (Fig. 2). Crystallization of SeMet-derivative protein was carried out using the same procedures as described for the native protein.

Figure 1
SDS-PAGE of purification of the recombinant *Ljd*-LDH protein. Lane 1, molecular-weight markers (labelled in kDa). Lanes 2–8 show the purification procedure of *Ljd*-LDH using Ni-NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, supernatant and pellet fraction after centrifugation of whole cell extract, respectively; lane 5, flowthrough from the Ni-NTA column; lanes 6 and 7, washes with 5 and 10 mM imidazole, respectively; lane 8, elution with 300 mM imidazole. The 6×His *Ljd*-LDH protein is indicated on the right side of the gel.

Figure 2
Hexagonal crystals of *Ljd*-LDH. Crystals of the best quality were crystallized using 28% (w/v) polyethylene glycol (PEG) 400, 100 mM Tris-HCl pH 9.0, 200 mM magnesium sulfate.

Table 2
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	Apo	SeMet
Beamline	7A (MXII), PAL	BL26B2, SPring-8
Wavelength (Å)	1.0	0.97919
Oscillation (°)	1	1
Total rotation range (°)	246	360
Space group	<i>P</i> ₃ ₁ ₂ ₁	<i>P</i> ₃ ₁ ₂ ₁
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 90.5, <i>c</i> = 157.8, α = β = 90.0, γ = 120.0	<i>a</i> = <i>b</i> = 94.2, <i>c</i> = 158.0, α = β = 90.0, γ = 120.0
Resolution (Å)	50.0–2.13 (2.17–2.13)	50.0–2.00 (2.03–2.00)
Total reflections	356502	994182
Unique reflections	41739	54787
Completeness (%)	97.8 (83.5)	98.8 (100.0)
<i>R</i> _{merge} † (%)	5.7 (29.4)	15.2 (89.4)
<i>CC</i> _{1/2} ‡ (%)	100 (100)	99.5 (31.1)
<i>I</i> / <i>σ</i> (<i>I</i>)	28.8 (2.4)	12.9 (2.9)
Multiplicity	8.5 (4.2)	18.6 (22.6)

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections. ‡ The correlation of one half of the observations to the other half by PHENIX (Afonine *et al.*, 2012; Karplus & Diederichs, 2012).

Figure 3
Electron-density map ($2F_o - F_c$) and model of *Ljd*-LDH after *RESOLVE*. The electron-density map ($2F_o - F_c$) and model of *Ljd*-LDH after *RESOLVE* were prepared using *PyMOL* (Schrödinger, <http://www.pymol.org>). Se atoms found by *SOLVE* are presented as yellow-coloured spheres.

4. X-ray analysis

The crystals were transferred to a cryoprotectant solution consisting of 28% PEG 400, 100 mM Tris–HCl pH 9.0, 200 mM magnesium sulfate, 30% (v/v) glycerol, removed with a cryoloop and flash-cooled by immersion in liquid nitrogen. The native data were collected to a resolution of 2.1 Å on the 7A beamline of the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea. All data were indexed, integrated and scaled together using the *HKL*-2000 software

package (Otwinowski & Minor, 1997). The crystal of *Ljd*-LDH belonged to space group *P*₃₁₂₁, with unit-cell parameters *a* = *b* = 90.5, *c* = 157.8 Å. Assuming that the asymmetric unit contains two molecules of *Ljd*-LDH, the crystal volume per unit protein weight (*V*_M) is 2.58 Å³ Da^{−1}, which corresponds to a solvent content of approximately 52.3% (Matthews, 1968).

SAD (single-wavelength anomalous dispersion) data were collected from a SeMet-derivative crystal on BL26B2 of SPring-8, Hyogo, Japan at a wavelength of 0.97919 Å. 16 of the 18 Se atoms in the asymmetric unit were identified using *SOLVE* (Terwilliger & Berendzen, 1999) at 2.0 Å resolution. The electron density was improved by density modification using *RESOLVE* (Terwilliger, 2000), resulting in 80% of the residues being automatically built (Fig. 3). After the crystallographic model building and refinement of the selenomethionine structure, we performed molecular replacement using the selenomethionine structure as a search model for the refinement of the selenomethionine-free structure. Refinement of the selenomethionine-free structure to 2.13 Å resolution is in progress. The data statistics are summarized in Table 2.

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