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Discovery and characterization of a thermostable D-lactate dehydrogenase from *Lactobacillus jensenii* through genome mining

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ABSTRACT

The demand on thermostable D-lactate dehydrogenases (D-LDH) has been increased for D-lactic acid production but thermostable D-DLHs with industrially applicable activity were not much explored. To identify a thermostable D-LDH, three D-LDHs from different *Lactobacillus jensenii* strains were screened by genome mining and then expressed in *Escherichia coli*. One of the three D-LDHs (D-LDH3) exhibited higher optimal reaction temperature (50 °C) than the others. The T_{50}^{10} value of this thermostable D-LDH3 was 48.3 °C, much higher than the T_{50}^{10} values of the others (42.7 and 42.9 °C) and that of a commercial D-lactate dehydrogenase (41.2 °C). The T_m values were 48.6, 45.7 and 55.7 °C for the three D-LDHs, respectively. In addition, kinetic parameter (k_{cat}/K_m) of D-LDH3 for pyruvate reduction was estimated to be almost 150 times higher than that for lactate oxidation at pH 8.0 and 25 °C, implying that D-lactate production from pyruvate is highly favored. These superior thermal and kinetic features would make the D-LDH3 characterized in this study a good candidate for the microbial production of D-lactate at high temperature from glucose if it is genetically introduced to lactate producing microbial.

1. Introduction

Lactic acid, a monomer required for the production of polylactic acid (PLA), is the major end product of carbohydrate fermentation by industrially important homofermentative lactic acid bacteria. Two isomers of lactic acid, the dextrorotatory (D-) and levorotatory (L-) isomers, can be produced by lactate dehydrogenases (LDHs). Optically pure isomers can be produced as separate products using chiral-specific D- or L-lactate dehydrogenase (D- or L-LDH) enzymes [1]. D-LDHs catalyze the NAD-dependent conversion of pyruvate to D-lactic acid and the reverse reaction [2].

L-LDHs have been thoroughly studied because L-lactic acid is widely used in food, cosmetics and medicine [3,4]. In contrast, there are few applications of D-lactic acid [5,6], and detailed research on D-LDHs has been comparatively neglected [7,8]. Increasing interest in stereocomplex PLA, comprising PDLA and PLLA, which has better properties than racemic PLA – such as increased melting point and impact strength – has boosted the production of optically pure D-lactic acid, which is required for the preparation of PDLA [9–11]. L-Lactic acid can be produced commercially by microbial fermentation at high yields and titers at temperatures between 30 and 40 °C [12,13]. However, despite the fact that several lactic acid bacteria, such as *Lactobacillus delbrueckii* and *Lactobacillus coryniformis*, produce D-lactic acid rather than L-lactic acid [14–16], the productivity of D-lactic acid fermentation using *Lactobacillus* was relatively lower than that of L-lactic acid fermentation at 40 °C incubation temperature, which may imply that thermostabilty of D-LDHs can be weaker than that of L-LDHs. Compared with thermostable L-LDHs [17], thermostable D-LDHs have not been well characterized at genetic level, and D-LDHs have lower thermostabilities than the L-LDHs in the same hosts [18,19]. Hyperthermostable D-LDHs have been found in thermophiles such as *Sulfolobus tokodaii*, but these enzymes cannot be used in the production of D-lactic acid because thermophilic enzymes are not highly active at normal culture temperatures (~40 °C) [20]. Wang et al. successfully produced D-lactate by microbial fermentation at 50 °C using a thermostable D-LDH [12]. However, this enzyme had evolved from a glycerol dehydrogenase, and it had dual activities toward glycerol and D-lactate. Therefore, the discovery or engineering of thermostable D-LDHs, which should be active at culture temperatures above 40 °C, is necessary for the economical production of D-lactic acid.

This work attempted to search for thermostable D-LDHs that could convert pyruvate into D-lactic acid. Even though the D-LDHs in supernatant of *Lactobacillus jensenii* culture broth were reported

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active at up to 50 °C [21], however there has been no available information for these D-LDHs including gene and protein sequence. We have tried the functional expression of genes encoding three *L. jensenii* D-LDHs in *E. coli* to find and characterize thermostable D-LDHs up to 50 °C.

2. Materials and methods

2.1. Phylogenetic analysis

Genes encoding D-LDHs were identified in these all *Lactobacillus* types which are *L. jensenii* 1153 (ABWG00000000), *L. jensenii* JV-V16 (ACGQ00000000), *L. jensenii* 27-2-CHN (ACOF00000000), *L. jensenii* 269-3 (ACOY00000000), *L. jensenii* SJ-7A-US (ACQD00000000), *L. jensenii* 115-3-CHN (ACQN00000000) and *L. jensenii* 208-1 (ADEX00000000) and their relatives in the GenBank database. The retrieved sequences were aligned, and the phylogenetic tree for D-LDH was generated using the maximum-likelihood method. The tree was evaluated using the bootstrap method with 1000 resamplings [22]. The alignment and the phylogenetic analysis were carried out using MEGA5 [23]. The analysis involved 27 amino acid sequences. All positions containing gaps and missing data were eliminated. The final data set comprised 328 positions. Three genes were selected as representative D-LDH genes from *L. jensenii*: ZP05866095 (D-LDH1 from *L. jensenii* SJ-7A-US), ZP05557096 (D-LDH2 from *L. jensenii* 27-2 CHN) and ZP04645201 (D-LDH3 from *L. jensenii* 269-3).

2.2. Construction of the expression plasmid

The genes for D-LDHs tagged with hexa-histidine at the C-terminus were chemically synthesized by GenScript (USA) in the pUC57 vector. The N-terminus was designed to contain an *Nde*I restriction site, and the C-terminus contained an *Eco*RI restriction site. The pUC57 vector containing the genes encoding D-LDHs was amplified using *E. coli* DH5α cells (RBC Bioscience, Taiwan) and isolated by using LaboPass™ Plasmid Mini Purification Kit (Cosmo Genetech, Korea).

The D-LDH genes were digested with the corresponding restriction enzymes and ligated with the *Nde*I-*Eco*RI-digested pET-22b(+) vector (Novagen, USA). The resulting plasmid containing the inserts was transformed into *E. coli* DH5α cells. The constructed pET-22b(+)-D-LDH1–3 vector was also transformed into an expression host, *E. coli* BL21 (λDE3) (RBC Bioscience, Taiwan).

2.3. Expression and purification of the D-LDHs

For small-scale experiments, *E. coli* transformants were picked from the plate and inoculated overnight in 5 mL of Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g sodium chloride per liter) supplemented with 100 μg/mL ampicillin for cells harboring pET-22b(+)-D-LDH1-3 Fresh LB medium (150 mL) with 100 μg/mL ampicillin was inoculated and incubated at 37 °C and 200 rpm. When the OD_{600} reached 0.6–0.8, the expression of the D-LDHs was induced for 4 h at 25 °C and 200 rpm by adding 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Cells were harvested by centrifugation (13,000 × *g*, 30 min and 4 °C), the supernatant was decanted, and the pellet was resuspended in BugBuster Master Mix (Novagen, USA) according to the manufacturer's guidelines. The resulting suspension was incubated at 25 °C for 30 min with gently shaking. The suspension was separated into soluble and insoluble fractions by centrifugation at 13,000 × *g* for 30 min at 4 °C.

Because D-LDH genes were fused with a hexa-histidine affinity purification motif (6× His-tag) at their C-termini, affinity chromatography (Ni-NTA column, Qiagen, USA) was used to purify the recombinant D-LDHs. Supernatants containing the soluble recombinant D-LDHs were incubated with 1 mL of Ni-NTA agarose bead (Qiagen, USA) at 4 °C for 60 min with gently shaking. The Ni-NTA agarose bead was loaded onto column after incubation, which were washed with 100 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole (pH 8.0)). The recombinant D-LDHs were finally eluted with 2 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole (pH 8.0)).

The expression and purity of the recombinant D-LDHs were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE. The SDS-PAGE was performed by the method of Laemmli using 5% stacking gel and 10% separating gel [24]. The same system was used for native PAGE except the SDS. 5% stacking gel and 10% separating gel was used as the resolving gel. After SDS-PAGE and native PAGE, the gels were stained by 0.1% Coomasie brilliant blue R-250 (Sigma–Aldrich, USA) in mixture of methanol/acetic acid/water, 45:10:45 (v/v), for 30 min and then destained in mixture of methanol/acetic acid/water, 10:10:80 (v/v), for overnight. The protein concentrations were determined using the Bradford reagent (Sigma–Aldrich, USA) according to the manufacturer's instructions.

The multimeric state of D-LDH3 was determined by measuring the apparent molecular weight using a Superdex™ 75 column and fast protein liquid chromatography (GE Healthcare, USA). The apparent molecular weight of D-LDH3 was calculated using molecular markers (Sigma–Aldrich, USA) including cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa) and amylase (200 kDa) in running buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl).

2.4. D-LDH activity assays

To determine the enzymes' activities, the absorbance at 340 nm was continuously recorded used to monitor the NADH concentration during the redox reactions catalyzed by the D-LDHs for 1 min. One unit of activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADH per minute under standard conditions (25 °C, pH 7.0). The determination of enzymes' activities for the reduction of pyruvate was performed using 50 mM Tris–HCl (pH 7.0) buffer solution containing 10 mM sodium pyruvate, 0.2 mM NADH, 0.1 μg of D-LDH1, 2 and 1 μg of D-LDH3.

To determine the enzymes' activities for the oxidation of sodium D-lactate, assay mixtures containing 50 mM buffer (Tris–HCl (pH 8.0) for D-LDH1, 2 and sodium bicarbonate–NaOH (pH 10.0) for D-LDH3), 100 mM sodium D-lactate, 2 mM NAD^+ and 10 μg D-LDHs were used. The reactions were started by the addition of the enzyme solutions.

2.5. Substrate specificity of D-LDHs

Substrate specificities of D-LDHs were examined using 50 mM sodium phosphate buffer (pH 8.0) containing 10 mM various substrates (sodium pyruvate, 2-ketobutyric acid, oxaloacetic acid, sodium phenylpyruvate) for reduction reaction and 10 mM various substrates (sodium D-lactate, (R)-2-hydroxybutyric acid, D-(+)-malic acid, D-(+)-3-phenyllactic acid) for oxidation reaction.

2.6. Thermostability and thermal inactivation of D-LDHs

For the study of thermal inactivation of D-LDHs at fixed constant temperature, the D-LDHs containing solutions were incubated at 45 °C for different time intervals from 0 to 90 min, and then cooled on ice for 10 min. Thermal inactivation constants of D-LDHs were determined by estimating values of half-life ($t_{1/2}$). All data analyses were performed by using linear regression fitting.

The kinetic stabilities of the D-LDHs were determined by measuring the residual activity after incubation at different temperatures. Enzyme solutions were incubated at 25–55 °C for 10 min and cooled for 10 min on ice before measuring the residual enzyme activities. Resistance to heat inactivation ($T_{50}{}^{10}$) is defined as the temperature at which half of an enzyme's activity remains after 10 min incubation relative to that after 10 min incubation at 25 °C.

Differential scanning fluorimetry (DSF) using a real-time quantitative PCR thermal cycler (LightCycler 480, Roche, USA) was utilized to determine the conformational stabilities of the enzymes by measuring the temperatures of their melting transitions (T_m). SYPRO Orange dye (Invitrogen, USA) was used to monitor enzyme unfolding. The hydrophobic dye could successfully detect the exposure of hydrophobic residues in the core during thermal unfolding [25]. A 100× stock solution was prepared by adding 20 μL of SYPRO Orange (5000× stock in DMSO) to 980 μL of phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Enzyme–dye solution (50 μL) containing 20 μL of purified D-LDH (10–50 μM), 20 μL of phosphate buffer and 10 μL of 100× SYPRO Orange stock (20× in the final solution) was placed in 96-well qPCR microplates and heated from 20 to 80 °C.

2.7. Determination of optimal temperature and pH for D-LDHs

The temperature and pH dependencies of D-LDHs were determined by using the standard activity assays for reduction and oxidation as described above. All parameters were kept constant except the temperature or pH. Assay solution without enzymes was preincubated at the fixed temperature from 10 to 60 °C for 60 min and reaction was initiated by the addition of enzyme to determine the optimal temperature. Relative activities were expressed respective to maximum activity. The optimal pH for D-LDH activity was assessed by varying the pH using standard potassium–citrate activity buffer (pH 5.0–6.0), Tris–HCl (pH 7.0–9.0) and sodium bicarbonate–NaOH (pH 10.0–11.0).

2.8. Kinetic parameters of D-LDHs

A kinetic study of D-LDHs was performed by measuring the oxidation and reduction rate for NADH and NAD^+ at pH 8.0 and 25 °C. Various concentrations of NADH (0.02–0.50 mM) were tested with pyruvate at a constant concentration of 50 mM. NAD^+ was tested at 0.02–2.00 mM with D-lactate at a constant concentration of 100 mM. The kinetic parameters (k_{cat} and K_m) were determined by Lineweaver–Burk plots. All experimental data in this study were the average values of triplicate measurements.

3. Results and discussion

3.1. Extraction of the D-LDH genes from the genome of L. jensenii and their amino acid compositions

The D-LDH genes of *L. jensenii* were clustered in two monophyletic groups in the phylogenetic tree (Fig. 1). Cluster I formed a monophyletic group with genes from *L. gasseri*, *L. johnsonii* and

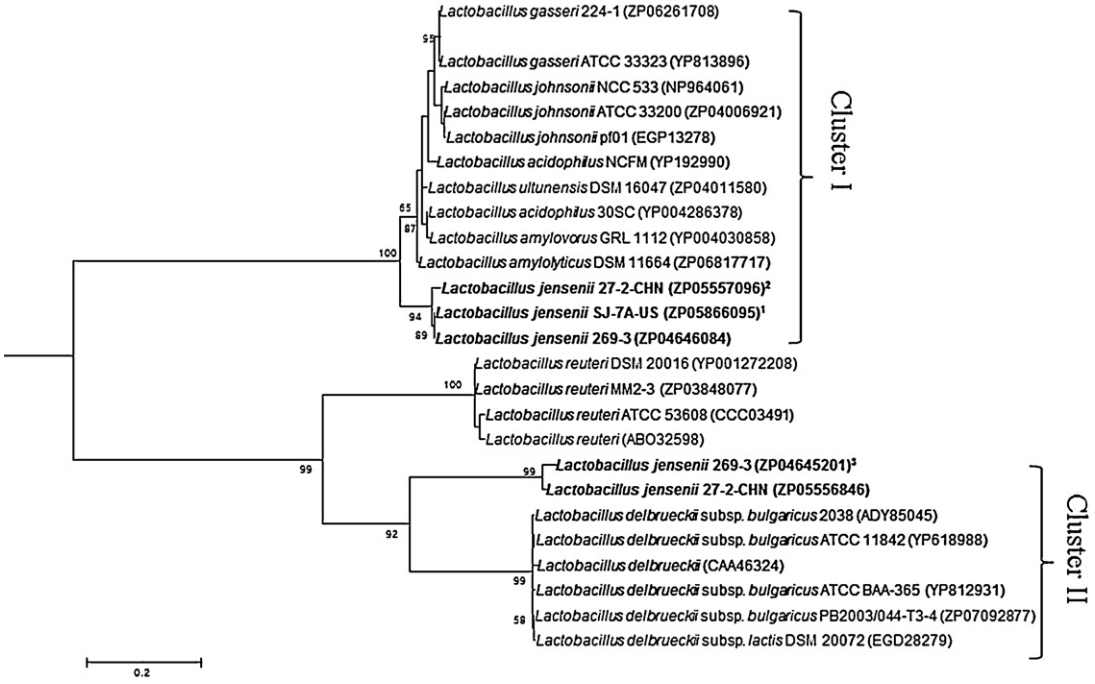

Fig. 1. The phylogenetic tree of D-LDH inferred using the maximum-likelihood method. The percentages at the nodes are the levels of bootstrap support based on maximum-likelihood analyses of 1000 resampled data sets. Only values over 50% are shown. The D-LDH gene of *Vibrio cholerae* V52 (ZP01680838) was used as an outgroup. Scale bar, 0.2 amino acid substitution per position. [1]D-LDH1; [2]D-LDH2, [3]D-LDH3.

L. acidophilus, and cluster II formed a monophyletic group with genes from *L. delbrueckii*, which can grow at 45 °C [26]. The similarity between D-LDH2 and D-LDH3 was only 47%, indicating that the genes in cluster I are paralogs of the genes in cluster II. Both the phylogenetic analysis and the levels of similarity between the D-LDH genes suggested that D-LDH3 likely has unique characteristics compared with D-LDH1 and D-LDH2.

Multiple sequence alignment was used to compare the D-LDH sequence identities (Fig. 2). The enzymes had conserved catalytic and NAD-binding residues. D-LDH1 and D-LDH2 showed 98% sequence identity. The sequence of D-LDH1 without first 20 amino acids at the N-terminus (MLHNKSCIIKISDCNIWRYN) is identical to that of D-LDH2 except for six amino acids (based on the numbering of D-LDH1; Met147 to Leu, Gln183 to Lys, Lys206 to Glu, Val265 to Ile, Lys291 to Glu and Gln294 to Lys). D-LDH2 and D-LDH3 had 33% sequence identity, and their amino acid compositions are quite different (Table 1). The comparison of D-LDH2 and D-LDH3 revealed that D-LDH2 contained more charged residues (30.33%; 9.31% Asp, 7.21% Glu, 2.40% His, 7.51% Lys, 3.90% Arg) and that D-LDH3 contained more hydrophobic residues (40.00%; 11.52% Ala, 7.27% Val, 8.48% Ile, 9.09% Leu, 3.64% Phe). Even though simple comparison based on the primary amino acid sequences and composition cannot the properties of folded enzymes, there are reports that hydrophobic residues in the core or at the protein–protein interface could contribute to greater thermostability of enzymes [27,28]. Moreover, D-LDH3 showed low sequence similarity with known mesophilic D-LDHs from *Lactobacillus helveticus* (PDB code: 2dld, 39%) and *Lactobacillus bulgaricus* (PDB code: 1j49, 35%) for which X-ray crystal structures were available. On the other hand, D-LDH1 and 2 are highly similar to mesophilic D-LDHs (80–90%), indicating that the high thermostability of D-LDH3 may the result of the sequence differences discussed above. Although no structural information is available, the amino acid compositions could be useful in explaining the differences in the thermostabilities of D-LDH1, 2 and 3.

3.2. Expression and purification of the D-LDHs

Specific activities of purified enzymes were calculated to be 3100–3150 U/mg for D-LDH1, 2 and 380 U/mg for D-LDH3 in the reduction of sodium pyruvate with NADH as a cofactor at 25 °C. And the purified D-LDH1 and D-LDH2 showed specific activities of 88–97 U/mg and the purified D-LDH3 showed specific activities of 42 U/mg in the oxidation of sodium D-lactate with NAD^+ as a cofactor at 25 °C.

A single band with an apparent molecular size of 34–43 kDa appeared, confirming the calculated molecular masses of 39.93, 37.73 and 37.41 kDa derived from the amino acid sequences of single subunits (Fig. 3a). However, the native PAGE gel (Fig. 3b) showed that D-LDH3 could have a multimeric form. Unlike other reports in BRENDA (http://www.brenda-enzymes.info/) stating that most D-LDHs can form homodimers, the size-exclusion chromatography analysis revealed that D-LDH3 could form a homotetramer (Fig. 3c). D-LDH1 and D-LDH2 seemed to form a mixture of oligomers and homodimers, respectively.

The enantiopreference of the D-LDHs was confirmed by the oxidation of D- and L- lactate. All enzymes isolated in this study exhibited D-LDH activity and no L-LDH activity (Table 2).

For reduction reaction catalyzed by various D-LDHs, D-LDH1 and D-LDH2 showed very similar substrate preference, on the while D-LDH3 showed different preference as shown in Table 3. Only D-LDH3 catalyzed the reduction of 2-ketobutyric acid considerably (54% of pyruvate reduction activity), however, other D-LDHs showed higher reduction activity for oxaloacetic acid (around 90% of pyruvate reduction activity). These results of D-LDH1 and 2 showed very similar substrate specificities of D-LDH from *L. bulgaricus* [29,30]. Morever, D-LDH from *L. bulgaricus* showed high sequence similarity with D-LDH1 and 2 as mentioned above. Even though oxidation activity of D-LDH3 showed much lower than that of reduction activity for pyruvate, D-LDH3 showed relatively high oxidation rate for (R)-2-hydroxybutyric acid, which implies that

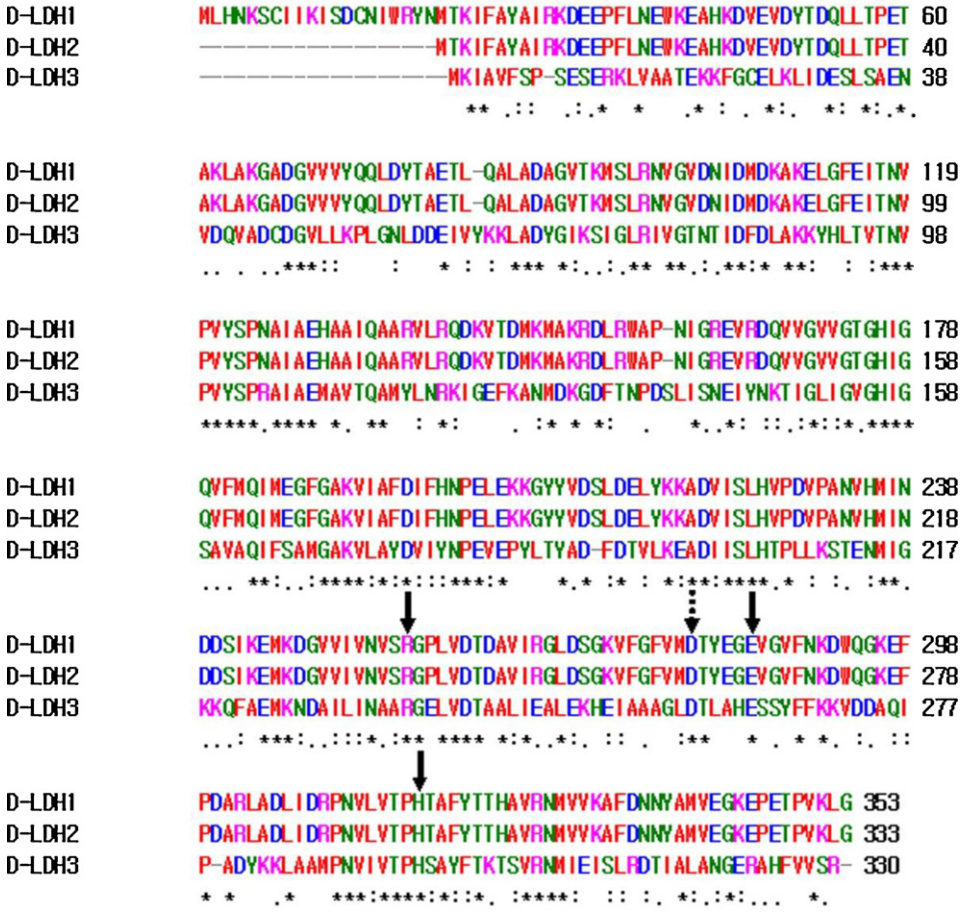

Fig. 2. Multiple sequence alignment of D-LDH1, 2 and 3. Asterisks and dots indicate identities and similarities, respectively. The solid and dashed arrows indicate conserved catalytic residues (Arg, Glu and His) and the NAD-binding residue (Asp) of the D-LDH family, respectively.

three dimensional structure of D-LDH3 can be quite different with those of other D-LDHs.

3.3. Optimum pH of the D-LDHs

D-LDHs from other hosts, such as *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* and *Staphylococcus epidermidis*, showed optimum pHs of 7.4–8.5 [30–32]. The optimum pH of the D-LDHs was determined by reduction reactions with NADH and oxidation reactions with NAD^+ (Fig. 4). D-LDH1, D-LDH2 and D-LDH3 showed similar activity–pH profile, and optimal activity was observed at pH 7 at reduction reactions. Maximal activities of oxidation reaction of D-LDH1 and 2 were obtained at pH 8. However, the optimum pH of D-LDH3 was shifted to a more basic pH (pH 10) (Fig. 4b). Garvie et al. reported that the D-LDH of *L. jensenii* has an optimum pH of 7.8 [33], similar to the values for D-LDH1 and D-LDH2. D-LDH3 had more alkaline pH optimum. Since D-LDH3 showed basic pH optimum for oxidation reaction compared with other D-LDHs, D-LDH3 can yield higher net reduction rate for pyruvate to produce D-lactate at neutral pH, which can be a leading point.

Table 1
Amino acid compositions of the investigated D-LDHs.

Amino acids	D-LDHs (number and percentage)		
	D-LDH1	D-LDH2	D-LDH3
Alanine	30 (8.50%)	30 (9.01%)	38 (11.52%)
Cysteine	2 (0.57%)	0 (0.00%)	2 (0.61%)
Aspartic acid	32 (9.07%)	31 (9.31%)	23 (6.97%)
Glutamic acid	22 (6.23%)	24 (7.21%)	22 (6.67%)
Phenylalanine	13 (3.68%)	13 (3.90%)	12 (3.64%)
Glycine	23 (6.52%)	23 (6.91%)	17 (5.15%)
Histidine	9 (2.55%)	8 (2.40%)	7 (2.12%)
Isoleucine	21 (5.95%)	18 (5.41%)	28 (8.48%)
Lysine	27 (7.65%)	25 (7.51%)	27 (8.18%)
Leucine	22 (6.23%)	22 (6.61%)	30 (9.09%)
Methionine	13 (3.68%)	11 (3.30%)	9 (2.73%)
Asparagine	18 (5.10%)	15 (4.50%)	16 (4.85%)
Proline	14 (3.97%)	14 (4.20%)	11 (3.33%)
Glutamine	10 (2.83%)	8 (2.40%)	5 (1.52%)
Arginine	14 (3.97%)	13 (3.90%)	9 (2.73%)
Serine	9 (2.55%)	7 (2.10%)	19 (5.76%)
Threonine	17 (4.82%)	17 (5.11%)	18 (5.45%)
Valine	41 (11.61%)	40 (12.01%)	24 (7.27%)
Tryptophan	4 (1.13%)	3 (0.90%)	0 (0.00%)
Tyrosine	12 (3.40%)	11 (3.30%)	13 (3.94%)

3.4. Optimal temperatures of the D-LDHs

The optimal temperatures of the D-LDHs were determined by measuring reduction reactions of sodium pyruvate as a substrate and oxidation reactions of sodium D-lactate as a substrate. Maximal activity of reduction reaction of the D-LDHs was observed at 50 °C. Only D-LDH3 was active above 55 °C (Fig. 5a). At 55 °C, D-LDH3 exhibited 80% of its activity at 50 °C, but the other D-LDHs exhibited less than 5% of their activities at 50 °C. In the oxidation reaction, only D-LDH3 was active above 40 °C (Fig. 5b). At 45 °C, D-LDH3 exhibited 85% of its activity at optimal temperature (40 °C), but the other D-LDHs exhibited less than 5% of their activities at optimal temperature (30 °C). Only D-LDH3 was active over a broad temperature range, and thus this enzyme could be a suitable D-LDH for the production of D-lactate at high-temperature cultures. It implies that D-LDH3 may have more rigid folding structure compared with those of other D-LDHs. Considering most D-LDH

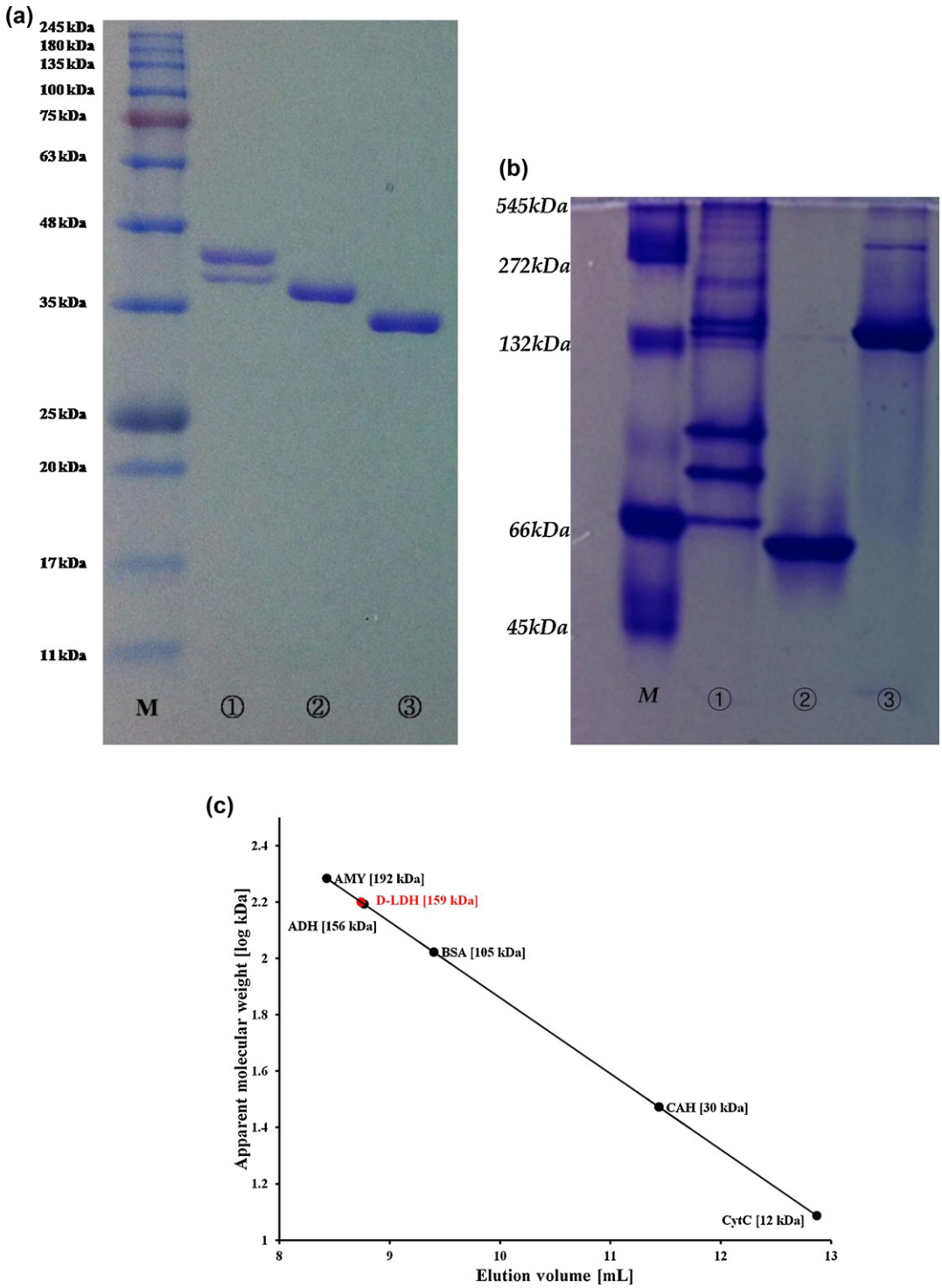

Fig. 3. Expression and purification of recombinant D-LDHs. (a) SDS-PAGE analysis: *Lane* M: molecular mass markers (GenDEPOT, USA), (1): purified D-LDH1 (0.5 μg), (2): purified D-LDH2 (0.5 μg), (3): purified D-LDH3 (0.5 μg). (b) Native PAGE analysis: *Lane* M: molecular mass markers (Sigma–Aldrich, USA), (1): purified D-LDH1 (10 μg), (2): purified D-LDH2 (10 μg), (3): purified D-LDH3 (10 μg). (c) The apparent molecular weight of D-LDH3 determined by the FPLC system. AMY: amylase, ADH: alcohol dehydrogenase, LDH: D-LDH3, BSA: albumin, CAH: carbonic anhydrase, CytC: cytochrome c.

showed low temperature as optimum, D-LDH3 can attract more attention since it can be used useful biocatalysts in vitro as well as in vivo [2].

3.5. Thermostability and thermal inactivation of D-LDHs

The thermal inactivation rate constants (k_d) of D-LDHs were determined from the slope of the logarithmic plot of activity against time (Fig. 6). The time required for the residual activity to be reduced to half ($t_{1/2}$) of D-LDHs at 45 °C was calculated to be 39.4, 47.6 and 72.2 min, respectively (Table 4). It means that D-LDH3 showed much longer lifetime to be thermally inactivated at 45 °C compared with other D-LDHs. In addition of the half time for inactivation, other parameters showing kinetic and thermodynamic stabilities also point out the superior property of D-LDH3 (Table 4).

Table 2
The enantioselectivity of the three D-LDHs toward D- and L-lactate.

Substrate	Specific D-LDHs activity [U/mg]		
	D-LDH1	D-LDH2	D-LDH3
L-Lactate	<0.01	<0.01	<0.01
D-Lactate	96.88	88.71	41.91

Resistance to heat inactivation ($T_{50}{}^{10}$) was determined to compare the kinetic stabilities of the D-LDHs. *Lactobacillus mesenteroides* D-LDH (Megazyme Ireland Ltd, Ireland) was also tested to allow comparison with a commercially available D-LDH. D-LDH3 was the most thermostable of the four tested D-LDHs (Fig. 7a). After 10 min incubation at 45 °C, D-LDH1 and 2 and the commercial D-LDH showed less than 20% residual activity, whereas D-LDH3 retained 70% of its activity. The $T_{50}{}^{10}$ values of D-LDH1 and 2 and the commercial enzyme were calculated to be 42.7, 42.9 and 41.2 °C, respectively. The $T_{50}{}^{10}$ value of D-LDH3 was calculated to be 48.3 °C. The NAD-dependent D-LDHs from other bacteria, such as *L. plantarum*, *L. acidophilus*, *L. fermentum*, *L. leichmannii* and *Staphylococcus haemolyticus* [31,33], were inactivated completely at 50 °C, indicating D-LDH3 is more thermostable than the previously reported NAD-dependent LDHs. In previous study, Gasser et al. reported that the D-LDH of *L. jensenii* 62G lost 25% of its activity after 5 min incubation at 50 °C. According to this study, this loss of activity at 50 °C also seems to occur for the D-LDHs of *L. jensenii* 62G that are homologous to D-LDHs 1 or 2 but not for the D-LDH homologous to D-LDH3, a result that is supported by similar optimum pHs of D-LDHs 1 and 2.

Table 3
The substrate specificity of the three D-LDHs.

Substrate	Enzyme activity [U/mg] (%)*		
	D-LDH1	D-LDH2	D-LDH3
Sodium pyruvate	3838 (100)	2997 (100)	348 (100)
2-Ketobutyric acid	68 (0.27)	41 (1.37)	189 (54.21)
Oxaloacetic acid	3151 (82.12)	2877 (96.00)	18 (5.05)
Sodium phenylpyruvate	7 (0.18)	9 (0.31)	28 (8.07)
Sodium D-lactate	17.82 (100)	14.97 (100)	9.74 (100)
(R)-2-Hydroxybutyric acid	0.05 (0.27)	0.05 (0.32)	10.24 (105)
D-(+)-Malic acid	0.00 (0.00)	0.00 (0.00)	0.11 (1.09)
D-(+)-3-Phenyllactic acid	0.00 (0.00)	0.00 (0.00)	3.70 (38.0)

* Values in parenthesis mean the relative values for sodium pyruvate and sodium D-lactate, respectively.

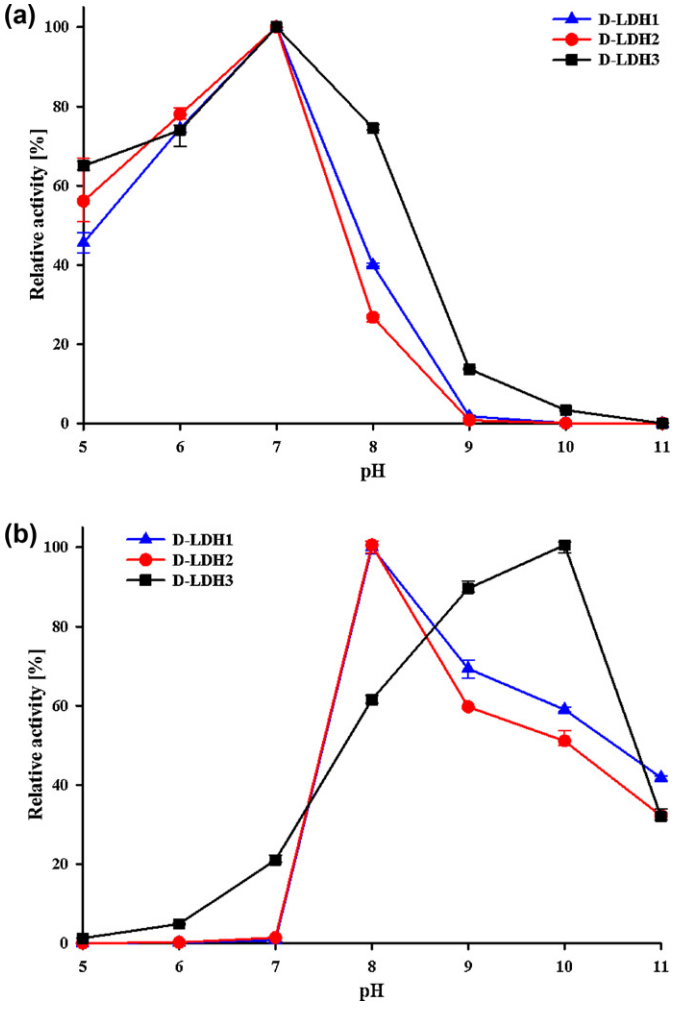

Fig. 4. The activity–pH profile of the D-LDHs measured at room temperature to determine optimal pH. (▲) D-LDH1, (●) D-LDH2, (■) D-LDH3. Relative activities were expressed respective to maximum activity. (a) The reduction reaction using sodium pyruvate as a substrate. (b) The oxidation reaction using sodium D-lactate as a substrate.

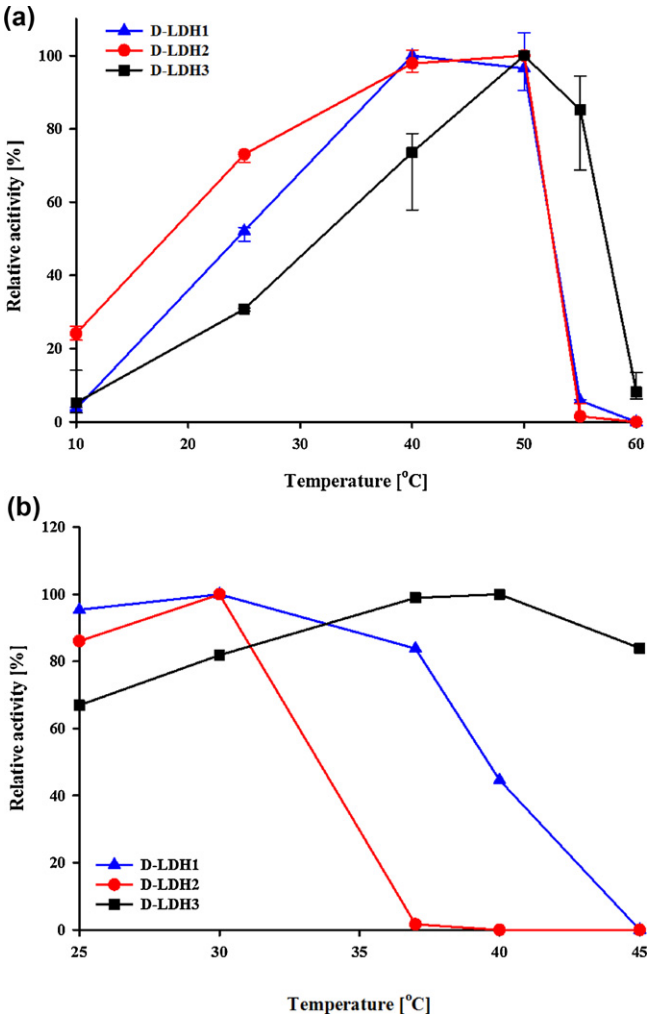

Fig. 5. The activity–temperature profile of the D-LDHs to determine optimal temperature. (a) The assay solutions without enzyme were pre-incubated at the indicated temperature for 1 h, and the activity was then measured at pH 7.0 using sodium pyruvate as a substrate for 1 min. (b) The enzymes and assay solutions were pre-incubated at the indicated temperature for 1 h, and the activity was then measured at pH 8.0 (Tris–HCl) for D-LDH1, 2 and at pH 10.0 (sodium bicarbonate–NaOH) for D-LDH3 using sodium D-lactate as a substrate for 1 min. Relative activities were expressed respective to maximum activity (▲) D-LDH1, (●) D-LDH2, (■) D-LDH3.

Table 4
Thermostabilities and thermal inactivation constants of D-LDHs.

	$T_{50}{}^{10}$ [°C]	T_m [°C]	K_d [min^{-1}]	$t_{1/2}$ [min]
D-LDH1	42.7	48.6	17.60×10^{-3}	39.4
D-LDH2	42.9	45.7	14.57×10^{-3}	47.6
D-LDH3	48.6	55.7	9.60×10^{-3}	72.2

The thermodynamic stabilities (T_m) of the D-LDHs were determined by the DSF method (Fig. 7c). D-LDH3 showed the highest thermodynamic stability among the recombinant D-LDHs. The calculated T_m values of D-LDHs 1, 2 and 3 were 48.59, 45.66 and 55.72 °C, respectively. Interestingly, D-LDHs 1 and 2 showed only single transition peaks during their thermal unfolding, but D-LDH3 had two transition peaks (51.45 and 55.72 °C, data not shown). During denaturation, the homo-tetrameric D-LDH3 first dissociated into monomers at 51 °C before each of the monomers underwent unfolding at above 55 °C. This structural analysis is consistent with the kinetic stability results of D-LDH3, showing that this enzyme has 40% residual active after 10 min incubation at 50 °C but is completely inactivated after 10 min incubation at 55 °C (Fig. 7a). Oligomerization can contribute to the thermostabilization of proteins, and hydrophobic interactions at protein–protein interfaces could aid oligomerization [34,35]. The homo-tetrameric state of D-LDH3 was an important structural feature contributing to its high thermostability, with its higher portion of hydrophobic residues possibly contributing to the multimerization. The reversibility of the heat-induced unfolding of the D-LDHs was investigated by cooling the denatured D-LDHs at 1.0 °C/min. All D-LDHs formed aggregates and exhibited no fluorescence changes (data not shown).

3.6. Determination of kinetic parameters

The kinetic parameters of the D-LDHs were determined using NADH and NAD^+ (Table 5). D-LDHs 1 and 2 had K_m values of 0.7 and 2.83 mM with NADH and 2.75 and 2.58 mM with NAD^+, respectively. D-LDH3 had K_m values of 0.036 mM with NADH and 0.41 mM with NAD^+. These affinities for cofactors were similar to those of other D-LDHs [8,36]. The catalytic efficiencies were much higher in the direction of sodium pyruvate reduction than in the direction of sodium D-lactate oxidation, suggesting that the enzymes catalyze sodium pyruvate reduction rather than sodium D-lactate oxidation.

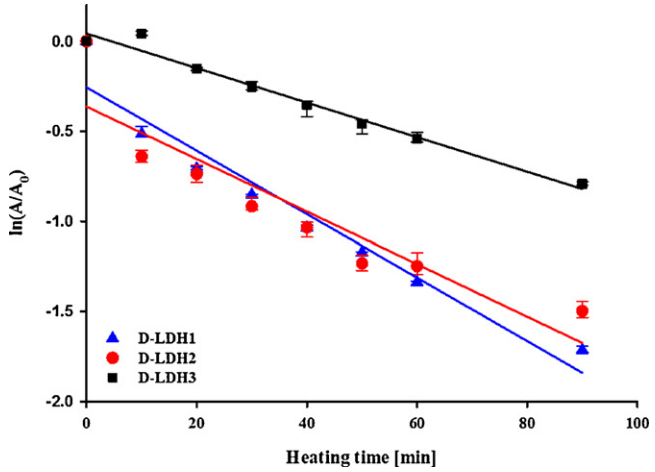

Fig. 6. The thermal inactivation of the D-LDHs. Residual activity was measured at 25 °C, pH 7.0, using sodium pyruvate as a substrate. (▲) D-LDH1, (●) D-LDH2, (■) D-LDH3.

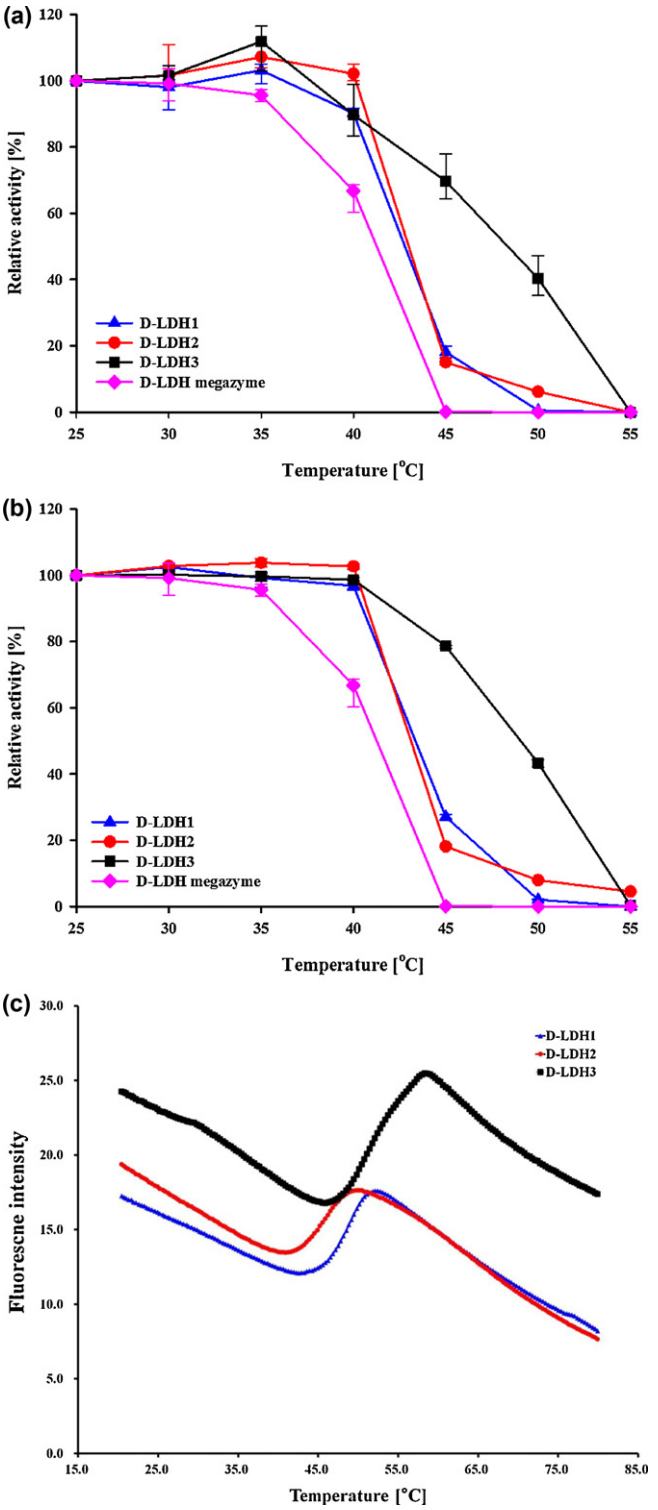

Fig. 7. The thermostability of the D-LDHs. (a) Kinetic stability. The enzymes were pre-incubated at the indicated temperature for 10 min and cooled for 10 min on ice. Residual activity was measured at 25 °C, pH 7.0, using sodium pyruvate as a substrate. (b) Kinetic stability. The enzymes were pre-incubated at the indicated temperature for 10 min and cooled for 10 min on ice. Residual activity was measured at 25 °C in Tris–HCl (pH 8.0) for D-LDH1, 2 and sodium bicarbonate–NaOH (pH 10.0) for D-LDH3, using sodium D-lactate as a substrate. (▲) D-LDH1, (●) D-LDH2, (■) D-LDH3, (♦) D-LDH from Megazyme. (c) Thermal unfolding curves of D-LDHs measured by the DSF method. (▲) D-LDH1, (●) D-LDH2, (■) D-LDH3.

Table 5
Kinetic parameters for reduction and oxidation.

Enzyme	Cofactor					
	NADH			NAD^+		
	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM^{-1} s^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM^{-1} s^{-1}]
D-LDH1	0.70	144.36	206.29	2.75	4.42	1.61
D-LDH2	2.83	516.42	182.57	2.58	3.65	1.41
D-LDH3	0.04	8.28	232.63	0.41	0.64	1.56

It is interesting that D-LDH3 had a lower turnover number than D-LDH1 and 2, which could be compensated for by a higher cofactor binding affinity. Therefore, D-LDH3 showed a higher catalytic efficiency in D-lactate production than D-LDH1 and 2. In addition, the relative catalytic efficiency of pyruvate reduction to D-lactate oxidation ($[k_{cat}/K_{m,reduction}]/[k_{cat}/K_{m,oxidation}]$) of D-LDH3 (149.12) was higher than that of D-LDH1 (128.13) and D-LDH2 (129.48), implying that D-LDH3 has the potential to efficiently produce D-lactate in other transformed hosts.

4. Conclusions

Three genes for D-LDHs were retrieved from the genome of *L. jensenii* and then successfully expressed in *E. coli*. The three enzymes, originating from the same species, were clustered into two different groups and showed different characteristics, suggesting that D-LDH genes with distinct activities can be found through genome-data mining, including the genomes of unculturable organisms or environmental metagenomes. The searching allowed the identification and subsequent characterization of a thermostable D-LDH, which could be used in the production of D-lactate monomer for the synthesis of PDLA and stereocomplex PLA bioplastics.

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