Appl Biochem Biotechnol (2010) 162:242–251
DOI 10.1007/s12010-009-8767-9

Bioconversion of Phenylpyruvate to Phenyllactate: Gene Cloning, Expression, and Enzymatic Characterization of D- and L1-Lactate Dehydrogenases from *Lactobacillus plantarum* SK002

Jianghua Jia · Wanmeng Mu · Tao Zhang · Bo Jiang

Received: 18 May 2009 / Accepted: 26 August 2009 /
Published online: 23 September 2009

Abstract Two DNA fragments containing the entire coding sequences of lactate dehydrogenase (LDH; *ldh*L1 and *ldh*D), whose enzymes have high activity for bioconversion of phenylpyruvate (PPA) to phenyllactate (PLA), were amplified from *Lactobacillus plantarum* SK002 using PCR. Sequencing showed open reading frames of 963 bp (*ldh*L1) and 999 bp (*ldh*D) encoding putative proteins of 320 and 332 amino acid residues, respectively. The LDH genes were cloned into an expression vector pET-22b(+) and expressed in *Escherichia coli* BL21(DE3). The purified recombinant L1-LDH and D-LDH had approximate (SDS-PAGE) molecular weights of 35 and 40 kDa, respectively. L1-LDH and D-LDH had PPA bioconversion specific activities of 71.06 and 215.84 U/mg with K_m values of 3.96 and 5.4 mM, respectively. The rL1-LDH and rD-LDH showed maximum enzyme activity at 30 and 40 °C while both had optimum activity at pH 6.0. L1-LDH exhibited a higher pH and temperature stability than D-LDH. The results show that the his-tagged *L. plantarum* SK002 D- and L1-LDHs are efficient catalysts for bioconversion of PPA to PLA.

Keywords Lactate dehydrogenase (LDH) · *Lactobacillus plantarum* SK002 · Phenyllactate (PLA) · Phenylpyruvate (PPA) · Gene cloning · Enzymatic characterization

Introduction

Phenyllactate (2-hydroxy-3-phenyl-propionic acid or PLA) is a novel antimicrobial compound with broad inhibitory activity against a range of food spoilage fungal and bacterial species (both gram positive and gram negative) [1, 2]. *Lactobacillus plantarum* has extensively demonstrated PLA-based antimicrobial activity in over 10 strains [3–5].

Previously, PLA was mainly produced from bioconversion of phenylallanine (Phe) through amino acid metabolism [6]. It was however shown that phenylpyruvate (PPA) is

J. Jia · W. Mu · T. Zhang · B. Jiang (✉)
State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu 214122, China
e-mail: bjiang@jiangnan.edu.cn

potentially a more suitable substrate for PLA production [7, 8]. Recently, lactate dehydrogenase (LDH) was demonstrated to be the main enzyme for bioconversion of PPA to PLA in *Lactobacillus* spp. [9]. Different isoforms of LDH have been studied in *Lactobacillus* spp. as well as across strains in order to find one suitable for commercial use [10, 11]. The two LDH isoforms, L-LDH (EC 1.1.1.27) and D-LDH (EC 1.1.1.28) however have different evolutionary origins, catalytic properties, substrate specificities, and the presence of the L- and D-LDH isoforms in *Lactobacillus* spp. widely varies between strains with some strains having one or both of L- and D-LDH [12, 13].

According to the *L. plantarum* WCFS1 genome [14], there are three lactate dehydrogenases genes (*ldh*D, *ldh*L1 and *ldh*L2) which may occur mutually exclusively. *L. plantarum* SK002, with a high output of PLA from PPA was selected in our study. Recently, we demonstrated that the product of *ldh*L2 (L2-LDH) in *L. plantarum* SK002 has capacity for bioconversion of PPA to PLA but with low activity and specificity [15]. In this study, the other two LDH genes (*ldh*D, *ldh*L1) from *L. plantarum* SK002 were cloned, expressed in recombinant *Escherichia coli* BL21(DE3)/pET-*ldh*L1 and *E. coli* BL21(DE3)/pET-*ldh*D and then characteristics of their bioconversion of PPA to PLA were assessed.

Materials and Methods

Strains, Plasmids, Enzymes, and Chemicals

L. plantarum SK002, with a high output of PLA from PPA, was isolated and kept in our lab frozen at −70 °C. The *E. coli* strains JM109 and BL21(DE3) (Shanghai Sangong Biological Engineering Technology and Services Co. Ltd, Shanghai, China) were used as host strains for genetic cloning and expression, respectively. Restriction endonuclease, T4 DNA ligase, Taq DNA polymerase, and isopropyl-1-thiobeta-D-galactopyranoside (IPTG) were obtained from Takara Biotechnology Co. Ltd (Dalian, China). The plasmid pMD-T (Takara Biotechnology Co. Ltd Dalian, China) and pET-22b(+) (Novagen, USA) were used for cloning and expression of the lactate dehydrogenase genes in *E. coli*. Ni Sepharose Fast Flow (1.0×10.0 cm) was obtained from Amersham Biosciences (Piscataway, USA) and electrophoresis reagents were purchased from Bio-Rad (California, USA). All other chemicals were of reagent grade and were obtained from the local commercial sources.

Amplification by PCR and Subsequent Cloning of *ldh*D and *ldh*L1 Genes

The genomic DNA of *L. plantarum* SK002 was obtained using the genomic DNA purification kit from Sangong (Shanghai, China) then used as a template for the polymerase chain reaction (PCR). The oligonucleotides were designed according to the nucleotide sequence of the lactate dehydrogenase genes of *L. plantarum* WCFS1. The oligonucleotides for *ldh*L1 incorporating *Nde*I and *Xho*I restriction sites were GCGCCCATATGTCAAG CATGCCAAATC (P1) and GCCGCCCTCGAGTTTATTT TCTAATTCAGC (P2), respectively. For *ldh*D, P1 and P2 incorporating *Eco*RI and *Xho*I respective restriction sites were CGGGGGAATTCATGAAAATTATTGCATAT and GCCGCTCGAGGTCAAACT TAACTTGCGTAT. The reaction mixtures for PCR contained 1× PCR buffer, each deoxynucleoside triphosphate (dNTP; 160 μM), each primer (0.4 μM), DNA template (1 ng/μl) and Taq DNA polymerase (1 U), all in a final volume of 20 μl. DNA amplification was started at 94 °C (5 min) and recycled 35 times as follows: 94 °C (60 s)

and 60 °C (60 s) for *ldh*L1 or 54 °C for *ldh*D (60 s), 72 °C (90 s), and a final extension at 72 °C (10 min). Verification of the PCR products was done using electrophoresis in 1.0% agarose gel stained with ethidium bromide. The DNA fragments of interest (963 bp for *ldh*L1 and 999 bp for *ldh*D) were purified by the agarose gel electrophoresis and then ligated into pMD-T cloning vector. The recombinant DNA was used to transform *E. coli* JM109. The clones harboring recombinant plasmids pMD-*ldh*L1 and pMD-*ldh*D were screened and verified by gene sequencing. The plasmids were isolated using the plasmid isolation kit from Sangong (Shanghai, China). The *ldh*L1 gene fragment was digested with *Nde*I and *Xho*I from pMD-*ldh*L1. The *ldh*D gene fragment, however, was digested with *Eco*RI and *Xho*I from pMD-*ldh*D. The *ldh*L1 or *ldh*D gene fragments were subcloned into the plasmid pET-22b(+) between *Nde*I or *Eco*RI sites and *Xho*I for both, to yield pET-*ldh*L1 or pET-*ldh*D, respectively. The LDH genes were expressed under control of T7 lac promoter. The recombinant *E. coli* BL21(DE3)/pET-*ldh*L1 or *E. coli* BL21(DE3)/pET-*ldh*D were finally constructed by transforming the pET-*ldh* into the host strain BL21(DE3) using the $CaCl_2$ method. The correct cloning of the LDH genes was confirmed in all cases both by restriction enzyme analysis and sequencing.

Expression and Purification of Recombinant D-LDH and L1-LDH

For expression of the recombinant LDHs, the transformed *E. coli* BL21(DE3) cells were grown in 400 mL of LB medium containing ampicillin (50 μg/ml) at 37 °C and 200 rpm. Induction was done at OD_{600}=0.3 with 0.4 mM of IPTG. Then the cells further grown for an additional 12 h at 16 °C 180 rpm to OD_{600}=1.0 to express active enzymes, and then harvested by centrifugation at 4 °C for 10 min at 10,000 rpm.

To purify the recombinant LDHs, the cell pellets were resuspended in 20 mM phosphate buffer (pH 7.4) to 0.1 of the original broth volume, disrupted by sonication at 4 °C (600 W, pulse on, 1 s; pulse off, 2 s) for 10 min and then the cell debris were removed by centrifugation (15,000 rpm, for 30 min at 4 °C). The cell free extract was applied onto a Ni Sepharose Fast Flow previously equilibrated with binding buffer (20 mM phosphate buffer, 500 mM NaCl, pH 7.4) [16]. Unbound proteins were washed out with washing buffer (20 mM phosphate buffer, 500 mM NaCl, 100 mM imidazole, pH 7.4). The recombinant LDHs were eluted from the column with elution buffer (20 mM phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.4). The eluted fractions were dialyzed overnight against 20 mM phosphate buffer (pH 7.4) at 4 °C then assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%). The purified LDHs were stored at 4 °C before usage.

Enzymatic Characteristics of Purified Recombinant D-LDH and L1-LDH

The activity of the LDHs towards PPA and pyruvate was routinely determined by measuring the rate of disappearance of NADH at 340 nm [9, 17]. The assay mixture contained in 100 mM potassium phosphate buffer (pH 6.5) 0.6 μmol of NADH, 19.6 μmol of PPA or 2.27 μmol of pyruvate and relevant amounts of enzyme (among the range of 50–300 U/l) in a total volume of 3.0 ml. The enzyme assay was performed at 30 °C, and 1 U of enzyme activity was defined as the amount of enzyme that catalyzes the degradation of 1 μmol of NADH per minute [9]. The effect of pH on the PPA to PLA bioconverting activity of the rLDHs was measured in 100 mM of citrate phosphate buffer (pH 2.0), sodium acetate buffer (pH 3.0–5.0), potassium phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (8.0–9.0), and glycine–NaOH buffer (pH 10.0). The pH stability of the

LDHs was tested by pre-incubating the enzyme at 30 °C for 60 min in different buffers at the indicated pH values. For determining the optimum temperature, enzyme activity was measured at different temperatures ranging from 20 to 70 °C. To examine the thermal stability, the enzyme was pre-incubated at temperatures ranging from 20 to 70 °C for different periods of time before assay at 30 °C as described previously. To determine the apparent Michaelis–Menten constant (K_m), enzyme activities using PPA and pyruvate were measured by varying the concentrations of each substrate under optimum conditions of pH and temperature. The K_m values of the LDHs were calculated by non-linear curve fitting using OriginLab software (Massachusetts, USA) [18, 19].

Reverse Phase HPLC Analysis of PLA and PPA

The product obtained from PPA reduction by purified recombinant L1-LDH and D-LDH was confirmed using reverse phase high-performance liquid chromatography (HPLC) equipped with an Agilent Zorbax XDB-C18 column (4.6×150 mm, 5 μm) using diode array detector (Agilent 1200 series) at 210 nm [9]. Briefly, for enzymatic production of PLA, 2 mM PPA, 1 mM NADH, and about 2,000 U/l purified enzyme were dissolved in 100 mM potassium phosphate buffer (pH 6.5) in a total volume of 1 ml. The reaction mixture was maintained at 30 °C for 1 h and then terminated by the addition of 1 M HCl. The reaction mixture (10 μl) was then loaded to HPLC. Elution was performed with methanol/0.05% TFA (solvent A) and water/0.05% TFA (solvent B) at 1 ml/min and A/B ratios of 10:90, 100:0, 100:0, and 10:90, with run times of 0, 20, 23, and 25 min, respectively.

Results and Discussion

Cloning and Sequence Alignment

The oligosaccharide primers for PCR were designed from the LDH genes (*ldh*L1 and *ldh*D) from *L. plantarum* WCFS1. Using the genomic DNA of *L. plantarum* SK002 as the template, specific DNA fragments matching the sizes of *ldh*L1 and *ldh*D were amplified (Fig. 1). The fragments were ligated into the pMD-T cloning vector, and the recombinant plasmids pMD-*ldh*L1 and pMD-*ldh*D sequenced. Open reading frames of 963 bp (*ldh*L1) and 999 bp (*ldh*D) encoding putative proteins of 320 and 332 amino acid residues were found, respectively.

The genebank accession numbers for the genes were FJ712707 (*ldh*D) and FJ712706 (*ldh*L1). Based on putative amino acids, L1-LDH and D-LDH had a homology of 18%. The putative D-LDH and L1-LDH had a homology of 14.95% and 54% with L2-LDH [15], respectively. This indicates that *L. plantarum* SK002 contains three distinct LDH isoenzymes. It confirms the presence of two *ldh*L genes in *L. plantarum* in addition to the *ldh*D gene. Given the low homology of *ldh*L1 and *ldh*L2 with *ldh*D, the two are probably of different evolutionary origin from the *ldh*D gene as suggested by Taguchi et al. [20]. The two *ldh*L genes were as a result of duplicating events as recently explained by Cristescu et al. [21].

Sequence comparison of *ldh*L1 and *ldh*D to those from *L. plantarum* WCFS1 respectively showed 99% similarities at DNA level for both. Comparison of the amino acid sequence encoded by *ldh*D to that of other *ldh*D genes whose enzymes convert PPA to PLA showed that the highest identity was with *L. plantarum* ATCC 8041 (95%, ac.

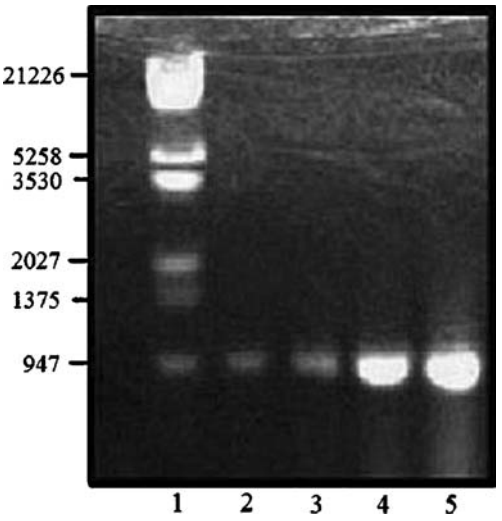

Fig. 1 PCR amplification of the *ldh*D and *ldh*L genes. *Lane 1* DNA standards, *lane 2* and *3* *ldh*D, *lane 4* nd *5* *ldh*L 1

BAA14352), *Lactobacillus delbruekii* (53.92%, ac. YP_812208), *Lactobacillus fermentum* (53.44%, ac. YP_001843448), *Lactobacillus acidophilus* (52.94%, ac. YP_192990), and *Lactobacillus brevis* (32.59%, ac.YP_794398). The *ldh*L1 gene on the other hand showed an identity similarity of 76.21% (ac. YP_794703), 63.88% (ac. YP_001576807), and 52.38% (ac. YP_193798) in amino acid sequence to L-LDH genes of *L. brevis*, *Lactobacillus helveticus*, and *L. acidophilus*, respectively.

Expression and Purification of the LDH Enzymes

The two enzymes were overexpressed using the pET-vector system under the T7 lac promoter in *E. coli* BL21(DE3) cells (Fig. 2). The enzymes were purified by centrifugation, cell sonication, and chelating using a Ni Sepharose Fast Flow as described above (Fig. 3).

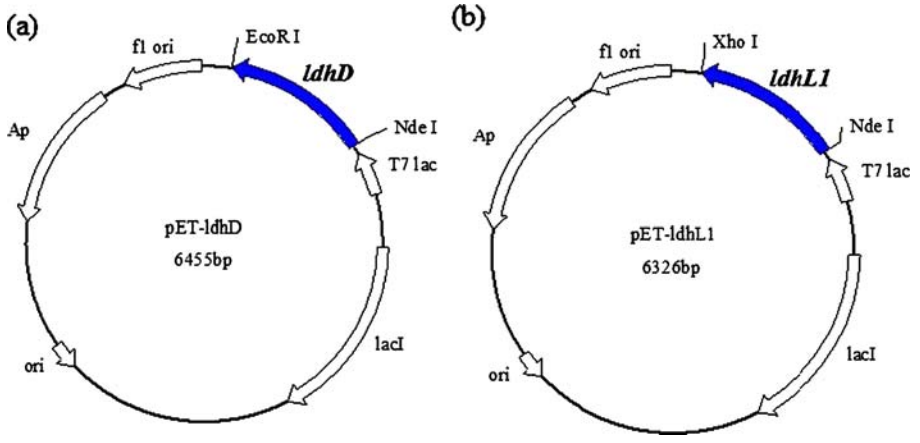

Fig. 2 Physical maps of expression plasmid pET-*ldh*D (**a**) and pET-*ldh*L1 (**b**) *T7 lac* T7 lac promoter, *ori* origin of replication, *lacI* the mutant repressor gene of lac operon, amplifier. The transcriptional direction of the LDH genes is indicated with *arrows*

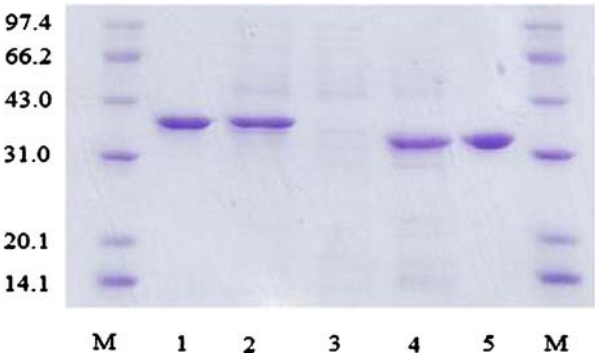

Fig. 3 SDS-PAGE analysis of the expression and purification of recombinant D and L1-LDH from *E. coli* BL 21 (DE3). *Lane M* standard protein molecular weight markers (kDa); *lane 1* and *5* purified recombinant D-LDH and L1-LDH, respectively; *lane 2* and *4* periplasm extract of induced transform ant harboring pET-*ldh*D and pET-*ldh*L1, respectively; *lane 3* periplasm extract if induced transform ant harboring the empty pET-22b(+) vector

The SDS-PAGE patterns of the his-tagged recombinant enzymes showed approximate molecular weights of 35 kDa (L1-LDH) and 40 kDa (D-LDH) which were similar to the predicted values based on the putative amino acid sequences.

Properties of Purified LDHs Catalyzing the Conversion of PPA into PLA

The L1-LDH showed a specific activity of 71.06 U/mg and 549.35 U/mg, while D-LDH demonstrated specific activity values of 215.84 U/mg and 2,682.14 U/mg for PPA and pyruvate, respectively. The ability of the purified enzymes to convert PPA to PLA was confirmed using reverse phase HPLC (Fig. 4). The specific activity of the recombinant L1-LDH on PPA was over 1,000 times that of L2-LDH reported earlier in *L. plantarum* SK002 [15]. The increase in activity is probably due to the low homology (54%) between them. The relatively low activity can further explain why there are no previous reports on the purification or separation of the two L-LDH isoforms in *L. plantarum*. The PPA to PLA bioconverting activity of the purified recombinant L1- and D-LDH were moderately high compared to other studies which have reported values ranging from a maximum of 593.404 U/mg for *L. helveticus* L-LDH [22] to a minimum of 0.06 U/mg for L2-LDH in *L. plantarum* SK002 [15]. The present study confirms the presence of three PPA to PLA bioconverting LDH isoforms in a single strain. *L. plantarum* therefore presents a formidable strain for assessing the evolutionary development of PPA to PLA bioconverting ability in *Lactobacillus* spp.

Few studies have assessed the pH dependence of the PPA to PLA bioconverting activity and stability of *Lactobacillus* spp. LDH. The variation of activity of the two purified enzymes with pH was assessed in the pH range of 3.0–8.0 with PPA as the substrate. Both enzymes showed optimum activity at pH 6.0 (Fig. 5a). A similar optimum was reported for LDH from *Lactobacillus* sp. SK007 [9]. The recombinant enzymes were both inactivated beyond pH 8.0. The pH-activity profile of the purified recombinant LDHs was in accordance that of the non-stereospecific in LDH studies in *Lactobacillus* sp. SK007 [9]. Purified recombinant L1-LDH however, showed a wider pH-activity range compared to both D-LDH and *Lactobacillus* sp. SK007 LDH [9]. It retained over 40% of its optimum activity at low pH (3.0) compared to D-LDH. The pH stability profile of purified

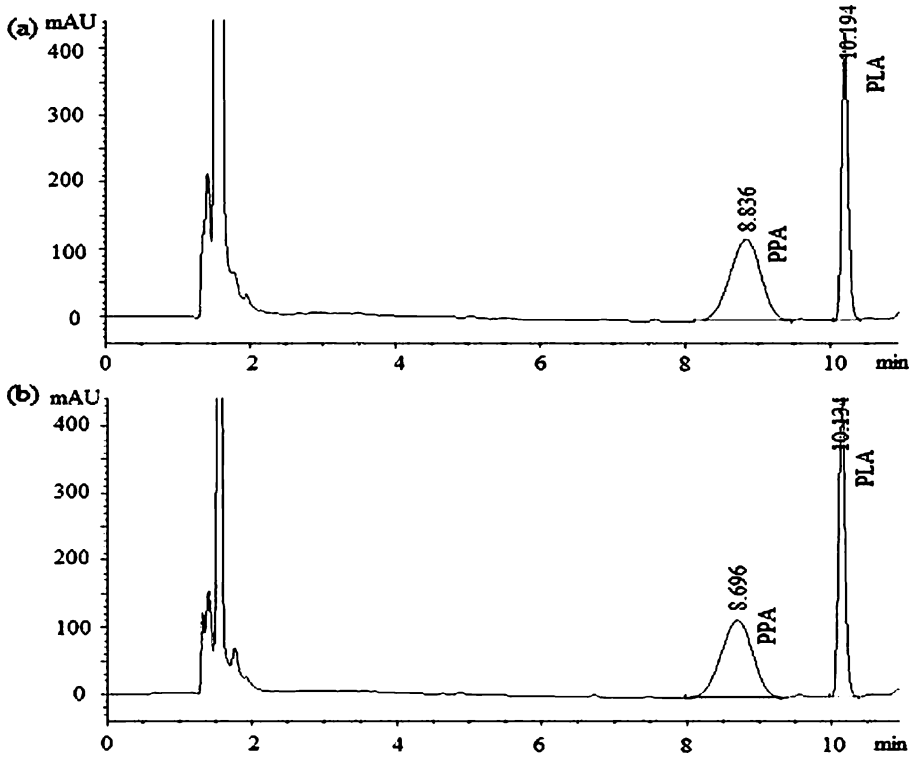

Fig. 4 RP-HPLC analysis of PPA bioconversation to PLA by purified recombinant D-LDH (**a**) and L1-LDH (**b**) enzymes, respectively

recombinant LDHs is shown in Fig. 6c. D-LDH rapidly lost its activity beyond the common optimum (pH 6.0) while L1-LDH was more stable (>80% activity, pH 3.0–9.0) like the *Lactobacillus* sp. SK007 LDH studied by Li et al. [9]. The variable pH stability of the two purified recombinant LDHs with in the same strain *L. plantarum* SK002, present

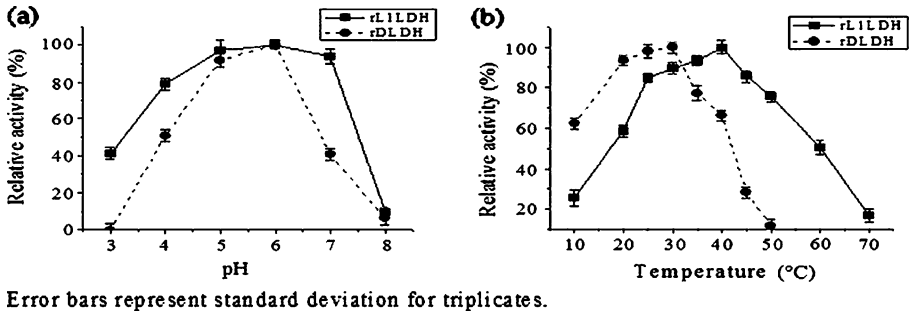

Fig. 5 Variation of purified recombinant D-LDH and L1-LDH activity with pH and temperature. **a** Effects of pH on the activity of recombinant D-LDH and L1-LDH. The activity assay was conducted at 30 °C in buffers of pH 3.0–8.0. The maximum activity at pH 6.0 was defined as 100%. **b** Effects of temperature on activity of recombinant D-LDH and L1-LDH. The LDH activity was measured in potassium phosphate buffer (pH 6.5) at 20–70 °C. The maximum activity at 30 and 40 °C for purified recombinant D-LDH and L1-LDH was defined as 100%, respectively

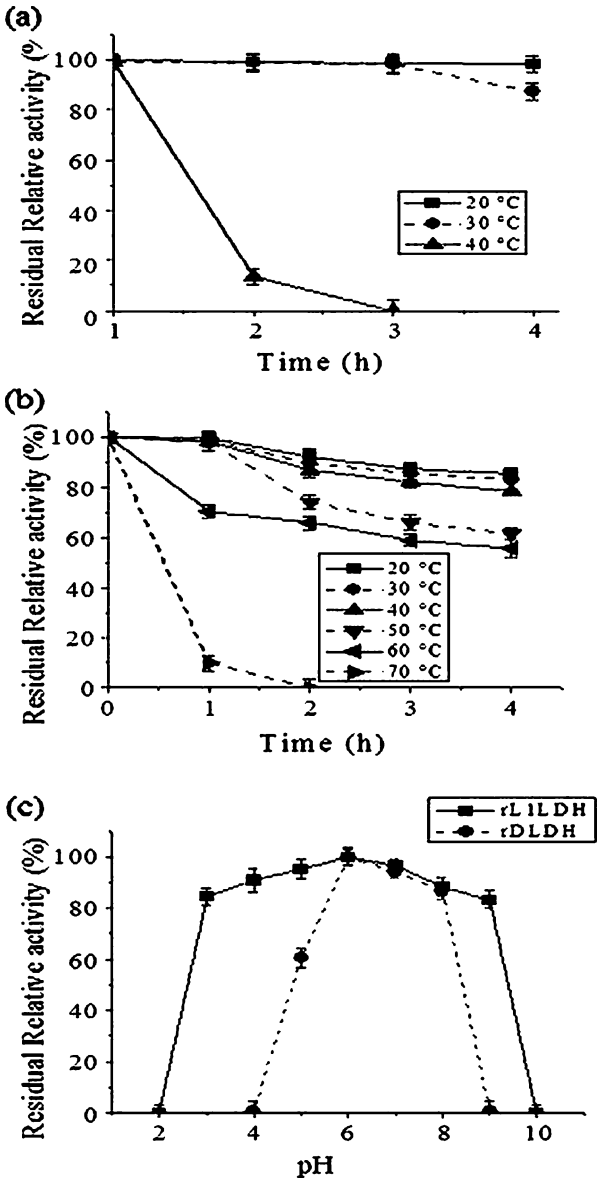

Fig. 6 Stability of purified recombinant D-LDH and L1-LDH Thermo-stability of the recombinant D-LDH (**a**) and L1-LDH (**b**). The enzyme was pre-incubated at 20–70 °C in potassium phosphate buffer (pH 6.5). An aliquot was drawn every 60 min then subjected to the activity assay at pH 6 (30 °C). The activity of untreated recombinant D-LDH and L1-LDH was defined as 100%. **c** Effect of pH on stability of recombinant D-LDH and L1-LDH. LDH activity was measured at 30 °C after incubation at 30 °C for 60 min in buffer of pH 2.0–10.0. The activity of untreated recombinant D-LDH and L1-LDH was defined as 100%

evolutionary implications (divergence based on environmental pH adaptation) and indicate that the L1-LDH has promise for industrial application in PLA production.

The temperature-activity profiles were assessed from 20 to 70 °C with PPA as the substrate (Fig. 5b). The optimum temperature for L1-LDH and D-LDH was 40 °C and

Table 1 Substrate specificities (K_m) for phenylpyruvate and pyruvate of D and L-LDH of some *Lactobacillus* species.

Strains	D/L-LDH	Pyruvate (mM)	PPA (mM)	Reference
L. plantarum SK002	D	0.06	5.4	This work
L. plantarum SK002	L1	0.23	3.96	This work
L. plantarum ATCC 8041	D	1.2	20	Taguchi et al. [20]
Lactobacillus sp. SK007	NS	0.32	1.69	Li et al. [9]
L. confusus 20196	D	0.68	3.0	Hummel et al. [17]
L. pentosus JCM1558	D	0.12	0.8	Tokuda et al. [23]
L. pentosus JCM1558	L	1.8	15	Arai et al. [24]

NS not shown

30 °C, respectively. Based on bioconversion of PPA to PLA, the optimum temperature in other studies has been reported as 45 °C for D-LDH in *Lactobacilus confusus* 20196 [17] and 40 °C for the non-stereospecific LDH in *Lactobacilus* sp. SK007 [9], thereby indicating a general variation around 40 °C. L1-LDH retained over 70% of maximum activity when held at 60 °C for 4 h (Fig. 6b). D-LDH however completely lost activity after incubation at 40 °C for 2 h (Fig. 6a). The purified L1-LDH was more stable than LDH for bioconversion of PPA to PLA reported by Li et al. [9]. The higher stability recombinant L1-LDH from the *L. plantarum* SK002 makes it a better candidate for industrial application.

The kinetic properties of the recombinant LDHs enantiomers were assessed using PPA and pyruvate as substrates. Both enzymes had a higher specificity for pyruvate than PPA with L1-LDH showing an apparent K_m value of 0.23 and 3.96 mM while D-LDH showed values of 0.06 and 5.4 mM for pyruvate and PPA, respectively. This was in agreement with previous studies (Table 1). Therefore for bioconversion of PPA to PLA, an appropriate LDH with a high PPA specificity and specific activity is required. The L1-LDH and D-LDH in this study are relatively good candidates. Notably, the *L. plantarum* SK002 D-LDH K_m values for pyruvate and PPA were 20- and fourfold lower than those reported by Taguchi et al. for *L. plantarum* ATCC 8041 [20].

In conclusion, there are two other enantioselective lactate dehydrogenase genes (*ldh*D and *ldh*L1) in *L. plantarum* SK002. The two genes have a homology of 18% based on the putative amino acid sequence. The two genes were overexpressed in recombinant *E. coli* BL21(DE3)/pET-*ldh*D and *E. coli* BL21(DE3)/pET-*ldh*L1, respectively. The specific activities of recombinant his-tagged L1-LDH and D-LDH for bioconversion of PPA to PLA were 71.06 U/mg and 215.84 U/mg, respectively. The characterization of enzymatic activities showed that the *L. plantarum* SK002 D- and L1-LDH exhibited same optimum pH for activity but varied significantly in specificity, temperature optima, temperature stability, and pH stability. These results show that the his-tagged *L. plantarum* SK002 D- and L1-LDHs are efficient catalysts for bioconversion of PPA to PLA hence good future candidates for genetic manipulation to improve industrial bioconversion of PPA to PLA.

Acknowledgement This work was supported by the grants from the National High Technology "863" Program of China, project No. 2006AA10Z334, the Natural Science Foundation of Jiangsu Province, project No. BK2008099 and BK2008003, the Research Program of State Key Laboratory of Food Science and Technology, Jiangnan University, project No. SKLF-MB-200804 and SKLF-TS-200805, and the Program for Innovative Research Team of Jiangnan University, project No. 2008CXTD01.

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