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Directed modification of L-*Lc*LDH1, an L-lactate dehydrogenase from *Lactobacillus casei*, to improve its specific activity and catalytic efficiency towards phenylpyruvic acid

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ABSTRACT

To improve the specific activity and catalytic efficiency of L-*Lc*LDH1, an NADH-dependent allosteric L-lactate dehydrogenase from *L. casei*, towards phenylpyruvic acid (PPA), its directed modification was conducted based on the semi-rational design. The three variant genes, *Lcldh1*^{Q88R}, *Lcldh1*^{I229A} and *Lcldh1*^{T235G}, were constructed by whole-plasmid PCR as designed theoretically, and expressed in *E. coli* BL21(DE3), respectively. The purified mutant, L-*Lc*LDH1^{Q88R} or L-*Lc*LDH1^{I229A}, displayed the specific activity of 451.5 or 512.4 U/mg towards PPA, by which the asymmetric reduction of PPA afforded L-phenyllactic acid (PLA) with an enantiomeric excess (ee_p) more than 99%. Their catalytic efficiencies (k_{cat}/K_m) without D-fructose-1,6-diphosphate (D-FDP) were 4.8- and 5.2-fold that of L-*Lc*LDH1. Additionally, the k_{cat}/K_m values of L-*Lc*LDH1^{Q88R} and L-*Lc*LDH1^{I229A} with D-FDP were 168.4- and 8.5-fold higher than those of the same enzymes without D-FDP, respectively. The analysis of catalytic mechanisms by molecular docking (MD) simulation indicated that substituting I229 in L-*Lc*LDH1 with Ala enlarges the space of substrate-binding pocket, and that the replacement of Q88 with Arg makes the inlet of pocket larger than that of L-*Lc*LDH1.

1. Introduction

NAD(P)(H)-dependent L-lactate dehydrogenases (L-LDHs, EC 1.1.1.27) can catalyze the oxidation-reduction between L-α-hydroxycarboxylic acids (R–CHOH–COOH) and α-ketocarboxylic acids (R–CO–COOH), whereas NAD(P)-independent ones (L-iLDHs) catalyze only the oxidation of L-α-hydroxy acids (Jiang et al., 2014). The majority of L-LDHs have been classified into two types: allosteric and non-allosteric L-LDHs. As the promising biocatalysts, L-LDHs have been widely applied to the industrial preparation of optically pure L-α-hydroxy acids, which are natural antiseptic agents for the preservation of food and feedstuff, and are also useful precursors for the synthesis of biodegradable polyesters, fine chemicals and pharmaceuticals (Zheng et al., 2013). For instance, L-PLA, which naturally exists in honey and wheat sourdough (Tuberoso et al., 2011; Ryan et al., 2009), could be produced through the asymmetric reduction of PPA by L-LDHs (Li et al., 2008). L-PLA is a versatile building block for the synthesis of highly value-added drugs, e.g., Englitazone, Statine, Danshensu, anti-HIV agents and L-phenylalanine (Zheng et al., 2011). Recently, it has been extended to the synthesis of poly(L-phenyllactic acid), a potential alternative of poly(L-lactic acid), with good hydrophobicity and toughness (Zheng et al., 2015).

To date, chemical synthesis, chemoenzymatic preparation, kinetic resolution and asymmetric reduction have been developed for the preparations of enantiopure α-hydroxy acids (Deechongkit et al., 2004; Chen et al., 2010; Das et al., 2010; Cha et al., 2007). Among them, the asymmetric reduction of α-ketocarboxylic acids by LDHs was a route available for selection owing to its many advantages, e.g., high enantioselectivity (ee_p > 99%), 100% theoretical yield, no or little by-product and environment-friendly process (Zheng et al., 2013). Unfortunately, the majority of known wild-type LDHs displayed lower specific activity and catalytic efficiency, especially towards substrates having large aliphatic or aromatic groups at C_α, making them unable to be used effectively. Therefore, it is necessary to excavate novel LDHs having high activity towards a broad spectrum of substrates, or to modify the primary and three-dimensional (3-D) structures of existing

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Table 1
PCR primers used for the site-directed mutagenesis of *Lcldh1*.

primer name	oligonucleotide sequence (5′→3′) [a]	size (bp)
Q88R-F	GGCGCTCCTCGGAAGCCTGGCGAAACTCGT	30
I229A-F	GCCGCTTATGAAATCGCCAAACTCAAGGGT	30
T235G-F	AAACTCAAGGGTGCCGGCTTCTATGGTATCGCAAC	35
Lcldh-R	CTCGAGCTGACGAGTTTCGATGTCATT	27

[a]The boxed CGG, GCC or GGC was used for the mutation of Q88, I229 or T235-encoding codon in *Lcldh1* into Arg, Ala or Gly-encoding one. The underlined base sequence indicated an *Xho*I site.

LDHs by protein engineering.

Reportedly, the several structural modifications of a thermostable L-LDH, designated *Bs*LDH, from *Bacillus stearothermophilus* were conducted by site-directed mutagenesis (Kallwass et al., 1992), DNA shuffling (Binay et al., 2009) and peptide segment substitution (Wilks et al., 1992) for improving its catalytic efficiencies towards α-keto-carboxylic acids having large side-chains, the non-natural substrates for LDHs. For example, to recognize benzoylformic acid, a double-mutant I227A/T233G (formerly I240A/T246G numbered by dogfish muscle LDH (Wigley et al., 1992)) of *Bs*LDH was rationally designed and obtained. The experimental results exhibited that I227A/T233G has low K_m and high k_{cat} towards benzoylformic acid (Binay et al., 2013). In another research, six mutant libraries of *Bs*LDH were created by site-saturation mutagenesis at residues sites of A (N85 and Q86), B (R92), C (R155), D (H179), E (I227) and F (T233). Two mutants N85C86 and N85R86 with markedly improved catalytic efficiencies towards benzoylformic and oxaloacetic acids, respectively, were selected from one library (Aslan et al., 2016). In addition, to improve the catalytic performance of *P. stutzeri* L-iLDH towards L-mandelic acid, it was re-designed based on the multiple sequence alignment of related L-iLDHs and the analysis of their active centre structures. The kinetic resolution of 40 g/L DL-mandelic acid was performed by engineered *E. coli* expressing L-iLDH's mutant (V108 A), retaining 20.1 g/L D-mandelic acid (ee_s > 99.5%) and producing 19.3 g/L benzoylfomic acid, simultaneously (Jiang et al., 2012).

A wild-type L-*Lc*LDH1-encoding gene, *Lcldh1* (GenBank accession no. MF582630), was amplified from *L. casei* CICIM B1192, and expressed in *E. coli* BL21(DE3). Similar as the vast majority of L-LDHs, L-*Lc*LDH1 exhibits high activity towards pyruvic acid, a natural substrate for L-LDHs, but low activity towards α-keto acids with large side-chains, such as PPA and oxaloacetic acid. In this work, referring to the reported findings about directed modifications of *Bs*LDH and based on the multiple sequence alignment of L-*Lc*LDH1 with *Bs*LDH and its mutants, Q88, I229 and T235 in L-*Lc*LDH1 were identified, and subjected to the site-directed mutagenesis of Q88R, I229A and T235G, respectively. Then, the specific activities and kinetic parameters of L-*Lc*LDH1 and its mutants were determined, and compared with each other. Finally, the mechanisms of mutants having remarkably improved specific activity and catalytic efficiency towards PPA were analyzed by MD simulation.

2. Materials and methods

2.1. Materials

E. coli BL21(DE3) and pET-22b(+) (Novagen, Madison, WI) were used for the construction of recombinant plasmids and expression of *Lcldh1* and its variants. Both pET-22b-*Lcldh1* and *E. coli*/*Lcldh1* were constructed in our lab. PrimeSTAR HS DNA polymerase and endonuclease *Dpn*I (TaKaRa, Dalian, China) were used for the site-directed mutagenesis of *Lcldh1*. *E. coli* BL21 and its transformants were cultured at 37 °C in the LB medium (g/L): tryptone 10, yeast extract 5 and NaCl 10, pH 7.2, and induced at the optimized temperature by 0.4 mM IPTG. PPA and D- or L-PLA were products of Sigma-Aldrich (St. Louis, MO). D-FDP trisodium salt octahydrate and NADH were purchased from Energy Chemical (Shanghai, China).

2.2. Selection of the residue sites in L-*Lc*LDH1 for site-directed mutagenesis

Using L-*Lc*LDH1 sequence as a template, other L-LDH ones, which shared more than 50% identity with that of L-*Lc*LDH1, were searched using the BLAST server at the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequence alignment between or among the related L-LDHs was carried out using the DNAMAN 6.0 software or Clustal W2 program (http://www.ebi.ac.uk.Tools/msa/clustalw2/).

L-*Lc*LDH1, efficiently catalyzing the asymmetric reduction of α-keto acids with small aliphatic groups, was similar as *Bs*LDH in sequence and substrate specificity. The crystal structure of *Bs*LDH (PDB code: 1LDN) was resolved by X-ray diffraction (Wigley et al., 1992). In the active centre of *Bs*LDH, four residues, R92, D152, R155 and H179, involved in substrate catalysis are absolutely conserved, and Q86, D181, I227 and T233 located in the substrate-binding pocket are also conserved, which were considered to be crucial for the specificity of L-LDHs towards L-lactic or pyruvic acid (Dunn et al., 1991; Wilks et al., 1988). To identify the residue sites in the substrate-binding pocket of L-*Lc*LDH1 corresponding to Q86, I227 and T233 of *Bs*LDH and the substituted residues, the multiple sequence alignment of L-*Lc*LDH1 with *Bs*LDH and its two mutants, *Bs*LDH M1 (Q86R) and M2 (I227A/T233G) with improved catalytic efficiencies separately towards oxaloacetic and benzoylformic acids (Wilks et al., 1988; Binay et al., 2013), was performed using the Clustal W2 program.

2.3. Construction of the variants of Lcldh1

The site-directed mutagenesis of the Q88-, I229- or T235-encoding codon in *Lcldh1* into the Arg-, Ala- or Gly-encoding one was carried out by whole-plasmid PCR (Sanchis et al., 2008). The PCR primers were designed and synthesized (Table 1). Using pET-22b-*Lcldh1* as the templete, the first round of PCR was carried out using a forward primer (Q88R-F, I229A-F or T235G-F) and a reverse primer (Lcldh-R) as the following conditions: a denaturation at 95 °C for 4 min, followed by 30 cycles of at 98 °C for 10 s, 53 °C for 30 s and 72 °C for 40 s. Then, the second round of PCR was conducted using first-round PCR product as primer: 30 cycles of at 98 °C for 10 s, 55 °C for 30 s and 72 °C for 5 min. Three target PCR products, pET-22b-*Lcldh1*^{Q88R}, -*Lcldh1*^{I229A} and -*Lcldh1*^{T235G}, were digested by *Dpn*I and transformed into *E. coli* BL2(DE3), respectively, constructing *E. coli* transformants, *E. coli*/*Lcldh1*^{Q88R}, /*Lcldh1*^{I229A} and /*Lcldh1*^{T235G}. Additionally, *E. coli* BL21(DE3) transformed with pET-22b-*Lcldh1* was used as a positive control (*E. coli*/*Lcldh1*), while with pET-22b(+) as a negative control (*E. coli*/pET-22b).