Highly stereoselective biosynthesis of (R)-α-hydroxy carboxylic acids through rationally re-designed mutation of D-lactate dehydrogenase

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An NAD-dependent D-lactate dehydrogenase (D-nLDH) of Lactobacillus bulgaricus ATCC 11842 was rationally re-designed for asymmetric reduction of a homologous series of α-keto carboxylic acids such as phenylpyruvic acid (PPA), α-ketobutyric acid, α-ketovaleric acid, β-hydroxypropionate. Compared with wild-type D-nLDH, the Y52L mutant D-nLDH showed elevated activities toward unnatural substrates especially with large substitutes at C-3. By the biocatalysis combined with a formate dehydrogenase for in situ generation of NADH, the corresponding (R)-α-hydroxy carboxylic acids could be produced at high yields and highly optical purities. Taking the production of chiral (R)-phenyllactic acid (PLA) for example, 50 mM PPA was completely reduced to (R)-PLA in 90 min with a high yield of 99.0% and a highly optical purity (≥99.9% e.e.) by the coupling system. The results presented in this work suggest a promising alternative for the production of chiral α-hydroxy carboxylic acids.

Enantiomerically pure α-hydroxy carboxylic acids are valuable chemicals and versatile building blocks for the synthesis of a variety of significant compounds with retention of chirality at C-2. 3-Phenyllactic acid (PLA), for instance, is a new type antiseptic agent and a useful precursor for the synthesis of drugs, including Danshensu (3,4-dihydroxyphenyllactic acid) that can inhibit platelet aggregation and coronary artery disease. Another optically active α-hydroxy carboxylic acid, α-hydroxybutyric acid, is an important material for the production of isoleucine, poly(α-hydroxybutyric acid), and some kinds of medicines, such as azinethrin in family of antitumour antibiotics. In addition, α-hydroxy-4-phenylbutyric acid is an important precursor to angiotensin-converting enzyme inhibitors. Thus, production of chiral α-hydroxy carboxylic acids has attracted researchers’ attention.

Many routes have been developed for the preparation of chiral α-hydroxy carboxylic acids, such as traditional chemical methods, chemoenzymatic routes, enzymatic kinetic resolution of racemic α-hydroxy carboxylic acids, and asymmetric bioreduction of α-keto carboxylic acids. Currently, asymmetric bioreduction of α-keto carboxylic acids by using dehydrogenases is the method of choice because of its excellent stereoselectivity, high theoretical yield up to 100%, and environment-friendly nature. However, the used dehydrogenases exhibited low activities, especially toward substrates with large aliphatic or aromatic groups at C-3. Therefore, there is still a requirement for new dehydrogenases that can reduce a broad spectrum of α-keto carboxylic acids with high efficiency. In addition, the used dehydrogenase is NADH-dependent, which reduces α-keto carboxylic acids into α-hydroxy carboxylic acids and oxidizes NADH into NAD⁺ in the meanwhile. Due to the high cost of the NADH, an in situ regeneration system of NADH should be introduced for its recycle. Formate dehydrogenase (FDH), which catalyzes the oxidation of formate to carbon dioxide while NAD⁺ is reduced to NADH, could be used in enzymatic synthesis of chemical compounds as a versatile biocatalyst for NADH regeneration.

Although candidate dehydrogenases can be isolated from the organisms in the environment or identified from genomic databases by genome mining, rational re-design of existing dehydrogenases that have been well characterized in crystal structures offers a promising alternative approach. Such a strategy has been successfully applied to xylose reductase and D-fructose-6-phosphate aldolase.
Based on this knowledge, the NAD-dependent d-lactate dehydrogenase (d-nLDH) of Lactobacillus bulgaricus ATCC 11842, an efficient d-lactic acid producing strain, has been extensively studied and its crystal structure is known. It was selected as a target to create new biocatalysts in this study. The resultant d-nLDH mutant exhibited high activity and high enantioselectivity toward α-hydroxy carboxylic acids with larger groups at C-3. Furthermore, d-nLDH mutant was combined with FDH from Candida boidiniiNCYC 1513 for the production of chiral (R)-PLA to evaluate the enzyme’s potential for industrial applications.

Results
Rational re-design of d-nLDH. d-nLDH exhibits high activity and stereoselectivity toward pyruvic acid and β-hydroxy pyruvic acid, but not toward bulkier substrate analogs. In order to improve the reduction activity of d-nLDH toward α-hydroxy carboxylic acids with larger groups at C-3, certain residues of d-nLDH, which are around the C-3 group of α-hydroxy carboxylic acids, should be re-designed. Previous studies of d-nLDH stereo structure revealed that Tyr52 and Phe299 are mainly responsible for hindering larger substrates because of their short distance from the side chain of α-keto carboxylic acids and their steric orientation. Moreover, Tokuda et al. have succeeded in converting the D-nLDH of Lactobacillus pentosus into d-α-hydroxyisocaproate dehydrogenase by replacing of Tyr52 with an aliphatic amino residue, Leu. Thus, these two residues, Tyr52 and Phe299, were selected for site-directed mutagenesis in this study. Tyr was substituted with Leu to abate the steric exclusion effect, and Phe was replaced by Tyr, which was considered to facilitate the binding of aromatic substrates. These d-nLDH Y52L and F299Y mutants were constructed and investigated as biocatalysts for the reduction of α-keto carboxylic acids (Fig. 1A) in the following study.

Expression and purification of d-nLDH and d-nLDH mutants. Using the primers ldhD1.f and ldhD1.r, ldhD was amplified by PCR from the genome of L. bulgaricus ATCC 11842. Using the primers ldhDY52L.f and ldhDY52L.r, TAC of L. bulgaricus was amplified by PCR from the genome of L. bulgaricus ATCC 11842. Using the primers ldhD1.f and ldhD1.r, TAC was replaced by TAC to obtain ldhDY52L. Using the primers ldhDF299Y.f and ldhDF299Y.r, TTC of L. bulgaricus was replaced by TTC to obtain ldhDF299Y. The ldhD(Y52L/F299Y) double mutant was constructed by combining the upper two steps. The DNA fragments were sequenced to confirm that only the expected mutation had occurred. Then, d-nLDH, Y52L mutant, F299Y mutant, and Y52L/F299Y double mutant were purified to homogeneity through the Ni-affinity chromatography (Fig. 1B).

Table 1 summarizes the results of specific activity determination of d-nLDH wild-type and mutants toward various α-keto carboxylic acids. The Y52L mutant exhibits robust activities toward the selected bulky substrates (3a to 9a), including aliphatic (longer than three carbons) and aromatic α-keto carboxylic acids. The specific activities are 16–1476-fold higher than those of d-nLDH wild-type. Furthermore, we found that the optimal substrate for this mutant switches to 4a, compared to the wild-type enzyme that is selective for 1a. Although the F299Y mutant is generally less active than the Y52L mutant, it also provides the improved activities toward α-keto carboxylic acids (Fig. 1A).

Substrate specificity and enantioselectivity of d-nLDH and d-nLDH mutants. Table 1 summarizes the results of specific activity determination of d-nLDH wild-type and mutants toward various α-keto carboxylic acids. The Y52L mutant exhibits robust activities toward the selected bulky substrates (3a to 9a), including aliphatic (longer than three carbons) and aromatic α-keto carboxylic acids. The specific activities are 16–1476-fold higher than those of d-nLDH wild-type. Furthermore, we found that the optimal substrate for this mutant switches to 4a, compared to the wild-type enzyme that is selective for 1a. Although the F299Y mutant is generally less active than the Y52L mutant, it also provides the improved activities toward α-keto carboxylic acids (Fig. 1A).
the substrates 4a, 6a, 7a, and 9a. Therefore, a Y52L/F299Y double mutant was constructed to investigate the combined effect of these two mutations.

The Y52L/F299Y double mutant also gives better activities toward the tested large substrates than the wild-type. However, its activities toward the tested substrates are weaker, compared to the Y52L mutant except for 8a and 9a. The optimal substrate of the Y52L/F299Y double mutant is substrate 8a. Table 1 shows that the Y52L/F299Y double mutant has a unique and robust activity toward substrate 8a (1.519 U mg⁻¹). This activity is higher than that of the wild-type enzyme toward its preferred substrate, 1a (771.4 U mg⁻¹). The most obvious distinction between the Y52L mutant and Y52L/F299Y double mutant is their activities toward substrates 1a and 2a. Although both the Y52L and F299Y mutants showed excellent activities toward 1a and 2a, the activity dramatically decreased in the context of the Y52L/F299Y double mutant.

To sum up, the Y52L mutant, which exhibits good activities toward all tested α-keto carboxylic acids, is superior to other mutants. Therefore, it is a good biocatalyst, suitable for the reduction of a homologous series of α-keto carboxylic acids with broad tolerance for the substitute at C-3. Enantiomeric excess (e.e.) values for the α-hydroxy carboxylic acids were also determined. The results indicate that the Y52L mutant retains its high stereoselectivity and produced the chiral (R)-α-hydroxy carboxylic acids with e.e. > 99.9% (Table 1).

Asymmetric reduction of phenylpyruvic acid (PPA) to (R)-PLA.

Asymmetric reduction of PPA (8a) was investigated in order to further explore the potential of D-nLDH mutants in the synthesis of important chiral (R)-α-hydroxy carboxylic acids. The resultant product, (R)-PLA (8b), can be used as a new type of natural antiseptic agent and as a key precursor for synthesis of hypoglyce-

Discussion

Enzymes have been widely accepted as useful biocatalysts for synthesis of a series of valuable organic compounds, especially their capacities for asymmetric catalysis. However, despite the widespread uses of enzymes in biosynthesis, the candidate enzymes with desirable characteristics are scarce. Many methods for exploitation and screening of the target enzymes have been reported in previous reports. Here, we demonstrated the utility of rational re-design to modify D-nLDH by using bioinformatics and gene cloning techniques. The resultant biocatalysts, D-nLDH mutants, possess good catalytic activities capable of transforming targeted substrates. The approach adopted in this study is efficient and the preconditioning is based on the structure and related catalytic mechanism of the objective enzyme. D-nLDH has been widely researched, including its property, characteristic, and structure. Based on these preconditions, Tyr52 and Phe299 were considered as pivotal residues for substrate spectrum and chosen for site-directed mutagenesis.

Enantiomerically pure α-hydroxy carboxylic acids are important synths for fine chemicals. Stereospecific nLDHs responsible for enantiomerically pure lactic acid production are of interest, as optical purity is the most common characteristic, and structure. Based on these preconditions, Tyr52 and Phe299 were considered as pivotal residues for substrate spectrum and chosen for site-directed mutagenesis.

Figure 2 | Asymmetric reduction of PPA (8a) with NADH cofactor recycling by using whole-cell system.
on non-natural substrates. Therefore, the mutants obtained in this study are attractive for production of α-hydroxy carboxylic acids. Because conversion of α-keto carboxylic acids to α-hydroxy carboxylic acids is accompanied by the oxidation of NADH to NAD, a cosubstrate is necessary to supply NADH. Therefore, the E. coli transformant coexpressing both ldhD mutant and fdh was constructed. In the absence of FDH, the reduction of 8a was slow and then stopped after the exhaustion of intracellular NADH (Fig. 4B). Thus, this process provides excellent bioreduction efficiency and high enantioselectivity for the production of α-hydroxy carboxylic acids.

In summary, the substrate selectivity of d-nLDH was successfully altered, and its activity toward substrates with large aliphatic or aromatic groups at C-3 was drastically improved. This study expands its range of application in the production of (R)-α-hydroxy carboxylic acids. More importantly, because of the high yield and high stereoselectivity of d-nLDH Y52L mutant, the whole-cell catalysis system containing d-nLDH Y52L mutant and FDH was successfully applied to the direct synthesis of (R)-phenyllactic acid. The method developed in this study could be used as a promising alternative for the production of highly optically pure α-hydroxy carboxylic acids. Our results for d-nLDH mutants might open up a way to reconstruct other enzymes, such as NAD-dependent l-lactate dehydrogenases and NAD-independent lactate dehydrogenases, based on their structures in order to modify the substrate spectra and improve the catalytic efficiency for the synthesis of valuable chiral compounds.

Methods

Chemicals. Pyruvate, β-hydroxypropionate, 2-oxobutyrate, phenylpyruvate, 4-hydroxyphenylpyruvate, racemic lactate, racemic 2-oxobutyroylactate, racemic 2-hydroxy-3-methylbutyrate, racemic phenyllactate, and racemic 4-hydroxyphenyllactate were purchased from Sigma-Aldrich. 3,3-Dimethyl-2-oxobutyrate, and racemic glycerate were purchased from TCI. 3-hydroxyphenyllactate were purchased from Sigma-Aldrich. 2-Oxovalerate, 2-hydroxy-3-methylbutyrate, racemic phenyllactate, and racemic 4-hydroxyphenylpyruvate, racemic lactate, racemic 2-hydroxybutyrate, racemic 2-hydroxypropionate, 2-hydroxy-3-methylbutyrate, and racemic mandelate were purchased from j&k Chemical. All other chemicals were of reagent grade.

Bacterial strains, plasmids and primers. Bacterial strains, plasmids, and oligonucleotide primers used in this study were listed in Table 3. Escherichia coli was grown at 37°C in Luria-Bertani (LB) medium and ampicillin was added at a concentration of 100 μg ml⁻¹, if necessary. L. bulgaricus ATCC 11842 was cultured in MRS media at 42°C and C. boidinii NCYC 1513 was incubated in YPD media at 30°C.

Cloning and site directed mutagenesis of ldhD. Genomic DNA of L. bulgaricus ATCC 11842 was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The ldhD gene was amplified using primers ldhD1.f and ldhD1.r with genomic DNA of L. bulgaricus ATCC 11842 as template and cloned into pmD18-T to construct pmD18-ldhD. Three ldhD mutants (ldhDY52L, ldhDf299Y, ldhDY52Lf299Y) were generated by using the TaKaRa MutanBEST Kit (Takara Biotechnology Dalian Co. Ltd., China) according to the manufacturer’s protocol. The point mutant at Tyr52 was introduced into primer ldhDY52LF and the point mutant at Phe299 was introduced into primer ldhDF299F. Plasmid pmD18-ldhD was used as template for single mutant construction and the resultant plasmid pmD18-ldhDY52L was used as template for double mutant construction. The mutants were confirmed by DNA sequencing.

Purification of d-nLDH and d-nLDH mutants. The resulting plasmids pmD18-ldhD, pmD18-ldhDY52L, and pmD18-ldhDF299 were digested with NotI and cloned into the NotI site of pETDuet-1 separately to construct four different expression plasmids, pETDuet-ldhD, pETDuet-ldhDY52L, pETDuet-ldhDF299, and pETDuet-ldhDY52LF299. The recombinant plasmids were separately transformed into E. coli BL21(DE3) for protein expression. Cells were
incubated aerobically in LB medium (100 μg ml⁻¹ ampicillin) at 37°C to an optical density of 0.6 to 0.8 at 600 nm. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression, and cultures were grown at 16°C for a further 10 h. Then, cells were harvested and suspended in a binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 20 mM imidazole [pH 7.4]) and disrupted by sonication. Thereafter, intact cells and cell debris were removed by centrifugation, and the resultant supernatant was filtered and loaded onto a HisTrap HP 5-ml column (GE Healthcare). Purification was performed with gradient elution by using 500 mM imidazole [pH 7.4]). All enzymes were purified to electrophoretic homogeneity for the activity assay.

### Biotransformation by whole cells coexpressing ldhD mutant and fdlh.

The plasmid pETDuet-ldhD<sup>Y52L</sup>-fdlh was constructed as follows: ldhD<sup>Y52L</sup> gene was amplified using primers fdh1.f and fdh1.r with genomic DNA of C. boidinii NCYC 1513 as template. The resulting PCR products ldhD<sup>Y52L</sup>-fdh were digested with NcoI-BamHI and Ndel-Xhol, respectively, and cloned into the MCS1 and MCS2 of pETDuet-1 to construct pETDuet-ldhD<sup>Y52L</sup>-fdlh, which was then transformed into E. coli BL21(DE3). Recombinant E. coli BL21(DE3) harboring pETDuet-ldhD<sup>Y52L</sup>-fdlh was cultured for protein expression and harvested for biotransformation. The reaction was carried out at 30°C and 120 rpm in phosphate buffer solution (PBS, 1/15 M [pH 7.4]) containing 50 g DCW l⁻¹ cells, 50 mM phenylpyruvate, and 100 mM sodium formate. The concentrations of phenylpyruvate and phenyllactate in the reaction mixtures were quantitatively analyzed by high-performance liquid chromatography (HPLC).

### Analytical methods.

The reduction activity of β-nLDH wild-type and mutants was assayed at 37°C in 1 ml of 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH, 10 mM β-keto carboxylic acids and the purified enzyme. The rate of NADH decrease in initial 1 min was determined by measuring the absorbance change at 340 nm. One unit of β-nLDH activity was defined as the amount that catalyzed the oxidation of 1 μmol NADH per minute. Protein concentration was determined by the Bradford method using bovine serum albumin for calibration.

The enantiomeric excess (ee) value of α-hydroxy carboxylic acids is defined as follows.

\[(R-α-hydroxy carboxylic acid) - (S-α-hydroxy carboxylic acid)\] \[(R-α-hydroxy carboxylic acid) + (S-α-hydroxy carboxylic acid)\]
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Author contributions

P.X., C.M., Z.Z. and C.G. conceived and designed the experiments. Z.Z., B.S., H.Z. and T.Q. performed the experiments. C.M., C.G. and P.X. contributed reagents and materials. C.M., P.X., C.M., Z.Z. and C.G. reviewed the manuscript.

Additional information

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