Modified catalytic performance of *Lactobacillus fermentum* L-lactate dehydrogenase by rational design

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**Abstract**

L-Lactate dehydrogenases can reduce alpha-keto carboxylic acids asymmetrically and generally have a broad substrate spectrum. L-Lactate dehydrogenase gene (LF-l-LDH0845) with reducing activity towards 3,4-dihydroxyphenylpyruvate and phenylpyruvate was obtained from *Lactobacillus fermentum* JN248. To change the substrate specificity of LDH0845 and improve its catalytic activity towards large substrates, site-directed mutation of Tyr221 was performed by analyzing the amino acids in the active center. Kinetic parameters show that the $k_{\text{cat}}$ values of Y221F mutant on 3,4-dihydroxyphenylpyruvate, 4-methyl-2-oxopentanoate, and glyoxylate are 1.21 s$^{-1}$, 1.35 s$^{-1}$, and 0.72 s$^{-1}$, respectively, which are 420%, 150% and 130% of the wild-type LDH0845. This study shows that the mutations of Y221 can significantly change the substrate specificity of LDH0845, making it become a potential tool enzyme for the reduction of alpha-keto carboxylic acids with large functional groups.

**Keywords** L-Lactate dehydrogenases · Single-point mutation · Substrate specificity · Rational design

**Introduction**

L-Lactate dehydrogenase (L-LDH, EC 1.1.1.27), an oxidoreductase of NAD$^+$ dependent L-specific hydroxy acid dehydrogenases family, exists in human beings, animals, plants and microorganisms in the form of tetramer (LDH-M4, LDH-M3H, LDH-M2H2, LDH-M3H, LDH-H4) [7]. Lactate dehydrogenase is an important enzyme in the glycolysis process. Under anaerobic conditions, LDH can catalyze pyruvate to lactic acid. When the animal lacks glucose, LDH can also oxidize lactic acid to pyruvate, which is converted into glucose through gluconeogenesis. LDH can be used as a biomarker for cancer diagnosis and prognosis. Therefore, LDH is considered as a promising target for the prevention and treatment of cancer [12]. The LDH0845 we screened earlier can be used as a bio-catalyst for the synthesis of many other $\alpha$-hydroxy carboxylic acids [24]. Generally, LDH has strong stereoselectivity and a wide range of substrates spectrum. LDH is not only used to produce lactic acid and phenylactic acid [23] but also can produce glycolic, mandelic acid and 3,4-dihydroxyphenyllactic acid. Those different alpha-hydroxy acids obtained were widely used in industrial production [39, 42, 45, 48].

L-Hydroxy acid dehydrogenases are kinds of homologous protein that use NAD(P)H as a cofactor to realize the stereospecific conversion of latent alpha-keto acids and chiral alpha-hydroxy acids [15]. The enzymes in the alpha hydroxy acid dehydrogenases family can be classified and named according to their optimal substrates. In this family, several enzymes that have been studied frequently include lactate dehydrogenase, malate dehydrogenase [13], glyoxylate reductase, 2-hydroxyglutarate dehydrogenase [4], 2-hydroxyisocaproate dehydrogenase [28] and malate dehydrogenase and a long chain alpha-hydroxy acid oxidase [16]. Generally, LDHs have a higher affinity for pyruvate. However, some known wild-type LDHs have high catalytic efficiency for other substrates with different side chains. For example, wild-type B$s$LDH has the
catalytic activity for 4-methyl-2-oxopentanoate with long side chains and phenylpyruvate with benzene ring [20]; L-LDHs derived from Clonorchis sinensis and Thermoanaerobacter ethanolicus JW200 has different catalytic efficiency for multiple substrates including pyruvic acid, 2-ketobutanoic acid, oxaloacetic acid, ketoglutaric acid, and so on [47, 50]. In addition, there are some other dehydrogenases that have better catalytic activity for larger side chain keto acids. For example, L-HicDH derived from Lactobacillus confusus has the ability to sterically reduce 2-ketocarboxylic acid [11]; AHADH derived from Trypanosoma cruzi has the value of $k_{cat}$ for p-hydroxyphenylpyruvate is 447 s$^{-1}$ [41]; SmHPPR, a hydroxyphenylpyruvate reductase from S. miltiorrhiza, has the value of $k_{cat}$ for 3,4-dihydroxyphenylpyruvate is 85 s$^{-1}$ [43]. Therefore, we can change the substrate specificity of enzymes through protein engineering to increase their affinity for different 2-oxo acids.

A lactate dehydrogenase gene, LF-L-LDH0845 (GenBank accession MG581696), was cloned from Lactobacillus fermentum JN248 in our laboratory. The enzyme can catalyze 2-ketobutanoic acid, alpha-ketoglutaric acid, 4-methyl-2-oxopentanoate, phenylpyruvate, benzoyl formic acid, p-hydroxyphenylpyruvate and 3,4-dihydroxyphenylpyruvate, with a relatively wide range of Substrate spectrum. LF-L-LDH0845 has an approximate molecular weight of 33.65 kDa [24]. In this study, LDH0845 and its mutants were modeled and docked with pyruvate and alpha-keto acids with larger functional groups in C3. Based on the analysis of residues around the substrate-binding pocket, the site-saturation mutagenesis of Tyr221 was determined. In addition, the enzymatic properties of 19 mutants were further studied to determine effect of implemented mutations on substrate specificity and activity.

Materials and methods

Materials

Strains, plasmids and culture conditions

Lactobacillus fermentum JN248 was a stock culture of our laboratory. E. coli JM109, E. coli BL21 (DE3) and pCold II vectors were purchased from TaKaRa (Dalian, China). Among them, E. coli JM109 was used for molecular cloning and propagation of cold shock expression plasmid pCold II, and E. coli BL21 was used for protein expression. All of the above were stored in the − 80 °C refrigerator. L. fermentum was activated and grown in MRS medium at 37 °C, and the other strains were grown in Luria–Bertani medium at 37 °C.

Tool enzymes and experimental reagents

Phenylpyruvate, p-hydroxyphenylpyruvate and nicotinamide adenine dinucleotide (NADH) were purchased from Aladdin (Shanghai, China), benzoyl formic acid was purchased from Urchem (Shanghai, China), isopropyl-β-D-thiogalactoside (IPTG) and ampicillin were purchased from Sangon Biotech (Shanghai, China). 3,4-dihydroxyphenylpyruvate was synthesized according to the method described in the literature [1]. FastPfu PCR SuperMix was purchased from Miozyme (Shanghai, China), Taq Master Mix was purchased from Novoprotein (Suzhou, China), and ABclonal MultiF Seamless Assembly was purchased from abclonal (Wuhan, China). BCA protein concentration determination kit was obtained from Solarbio (Beijing, China). Restriction endonucleases (SacI, BamHI), QuickCutTM DpnI, plasmid mini preparation kit, Plasmid Purification Kit and DNA purification kit were purchased from TaKaRa (Otsu, Japan). Color pre-stained standard protein Marker was purchased from Adamas (Shanghai, China). All of the above chemical reagents are analytical grade.

Methods

Homology modelling and molecular docking of LDH0845

The crystal structure of L-lactate dehydrogenase (LpLDH, PDB:1EZ4) derived from Lactobacillus pentosus was selected as the template, which was 67.63% identical to the primary structure of LDH0845 (Uchikoba et al., 2002). The Swiss model program (https://swissmodel.expasy.org/interactive) was used for the three-dimensional modeling and optimization of LDH0845 and its mutants [2, 3, 14, 38, 44], and then SAVES v6.0 (https://saves.mbi.ucla.edu/) was used to evaluate the modeling results [5, 25]. LDH0845 and its mutants obtained by modeling were used as receptors, small molecule substrates and NADH were used as ligands, docking was achieved through AutoDock and the results were operated in PyMOL software for visual observation. In addition, the sequence alignment between LDH0845 and several dehydrogenases (BsLDH, LcLDH, CNLDH, PTLDH, HicDH, AHADH, SmHPPR) with better catalytic activity for larger side chain ketoacids was performed using CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and ESPript 3.0 (https://esricht.ibcp.fr/ESPRiPT/ESPRiPT/).

Construction of recombinant plasmid

The primers were designed according to multiple cloning site of pCold II (Supplementary Table 1), and the LDH0845 gene was amplified from L. fermentum JN248. The plasmid pCold II was digested with Restriction endonucleases BamHI and Sac I, and then purified using a purification kit.
Afterwards, the target gene was ligated to pCold II with ABclonal MultiF Seamless Assembly ligase to construct a recombinant plasmid.

**Site-directed saturation mutagenesis of Tyr221**

Based on the analysis of the results of molecular docking and multiple sequence alignment, Arg 90, Tyr 221, Ile 224 and Thr 230 in the active pocket were replaced by alanine by primers and Whole Plasmid PCR technique (Supplementary Table 2). According to the alanine scan, it was decided to design mutation primers for site-directed mutagenesis of Tyr221 in l-LF-LDH0845 (Supplementary Table 3). The recombinant plasmid containing the wild-type l-LF-LDH0845 gene was used as template, and the primers were used for full-plasmid PCR [32]. In water bath at 37 °C, the PCR products were digested with Dpn I for 1 h to eliminate the methylated template. The digested product was then transformed into *E. coli* JM109, and transformants were selected and sent to the company (genewiz, Suzhou, China) for verification of further DNA sequence by Colony PCR and sequencing. The correctly mutated recombinant plasmids were transformed into *E. coli* BL21 for inducing expression to obtain the target protein.

**Expression and purification of l-LF-LDH0845**

*E. coli* BL21 were inoculated into Luria–Bertani medium with ampicillin (100 mg/mL), and cultured overnight at 37 °C, 220 rpm. The culture was inoculated into 50 mL Luria–Bertani medium containing ampicillin (100 mg/mL) at a volume ratio of 2%. It was shaken in a shaker at 37 °C and cultivated until the value of OD<sub>600</sub> was approximately 0.6-0.8. It was stably cultured at 15 °C for 30 min for pre-cooling. Later, IPTG was added to the final concentration of 0.4 mM, and then it was incubated for 24 hours at 15 °C, 200 rpm [31]. The bacterial solutions were centrifuged at 4 °C, 8000×g for 10 min, and the supernatants were discarded to collect the cells. The precipitates were suspended in phosphate buffer (pH 6.0) and sonicated (The power is 200 W, the time is 20 min, Ultrasound is turned on for 2 s, Ultrasound is turned off for 2 s) to break the cells, and then centrifuged again to remove cell debris, and the supernatants were crude enzyme solutions.

The crude enzyme solutions were filtered through 0.22 μm membrane and then separated and purified on the AKTA Avant chromatography system using HisTrapTM HP 5 mL column. The Ni<sup>2+</sup> affinity column was equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4), and then His-tagged recombinase was treated by linear elution with washing buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The eluted proteins were desalted to remove imidazole through a 5 mL HisTrap desalting column. The purified recombinant enzymes were analyzed on 12% SDS-PAGE and the concentrations of purified proteins were measured using the BCA protein concentration determination kit [30, 49].

**Activity assay and substrate specificity of l-LF-LDH0845 mutant**

The enzyme activity of LDH0845 was determined based on the change in absorbance of NADH at 340 nm [29]. The absorbance was measured using a spectrometer (Pristmatic Technology 759S UV–Vis Spectrophotometer) produced by Lengguang Tech (Shanghai, China). Eleven carboxylic acids with ketone carbonyl or aldehyde groups were selected as substrates, including pyruvate, 3,4-dihydroxyphenylpyruvate, p-hydroxyphenylpyruvate, 3-methyl-2-xybytic acid, alpha-ketoglutaric acid, phenylpyruvate, oxaloacetic acid, 2-ketobutyric acid, 4-methyl-2-oxopentanoate, benzoyl formic acid and glyoxylate (Supplementary figure 1). NADH was used as coenzyme to prepare 3 mL reaction system, which included 0.13 mM NADH, 0.5 mM substrate and 20 mM phosphate buffer (pH 6.0). The mixture was incubated at 25 °C for 15 min, and then an appropriate amount of enzyme solution was added to detect the change of absorbance. The amount of enzyme required for catalytic oxidation of 1 μmol NADH per minute under the above conditions was defined as a unit of enzyme activity (U), and the number of enzyme activity units per milligram of enzyme protein was defined as specific activity (U/mg). The enzyme activity was determined according to the above method. The enzyme activity of the wild-type enzyme was defined as 100%, and the enzyme activity of the mutants was calculated as its relative value. The substrate specificity of Y221 mutants was analyzed by drawing radar map based on calculation results.

**Characteristics of Y221F mutant**

The reaction system composed of disodium hydrogen phosphate-sodium dihydrogen phosphate (pH 6.0) was placed at different temperatures (10–50 °C) for 15 min, and the enzyme activity was measured to determine the optimal temperature. Buffers with different pH values (3.0–9.0) were configured, including disodium hydrogen phosphate-citrate buffer with a pH range of 3.0–5.5, and disodium hydrogen phosphate-phosphate with a pH range of 5.5–8.0, sodium dihydrogen buffer and sodium carbonate-sodium bicarbonate buffer with pH 9.0. After the reaction system was placed at the optimal temperature (25 °C) for 15 min, the absorbance is measured to determine the optimal pH. The enzymes were placed at different temperatures for 1 h, and the enzyme activity was measured under the optimal conditions (pH 6.0)
to determine the temperature stability. The enzyme solutions were placed at different pH for 1 h, and the enzyme activity was measured under optimal conditions (25 °C) to determine the pH stability. The enzyme activity determination method is the same as 2.2.5. The enzyme activity of the untreated enzyme solutions was defined as 100%, and the enzyme activity of the enzyme solutions treated by other conditions was the relative value. Substrates of different concentrations (0.25–7 mM) were added to the reaction system and placed in the optimal conditions (25 °C, pH 6.0) for 15 min. The enzyme solution with a protein concentration of 0.56 mg/mL was appropriately diluted and added to it, and the absorbance measurement and enzyme activity calculation were performed according to the methods mentioned in 2.2.5. Finally, the kinetic parameter data was calculated by the nonlinear fitting (the category is Growth/sigmoidal, the function is Hill, n = 1) of Origin2010 software.

Results

Homology modelling and mutation site design of LDH0845

After the modeling result is verified (Supplementary Note 2), NADH and natural substrate pyruvate were docked with LDH0845. The docking results are shown in Fig. 1. Several important residues around the substrate active pocket were the same as those of LpLDH, including residues Leu149, Arg153, and His177, which are involved in catalysis and substrate binding, as well as the residues Ala220, Tyr221, Ile224 and Thr230. Among them, R153 forms a dihydrogen bond with the carboxyl group of pyruvate through the guanidine group to facilitate the correct binding of the substrate at the catalytic site, H177 acts as a proton donor/acceptor to transfer hydrogen atoms in redox reactions [17, 26, 35]. Ile224 is close to the side chain of the substrate, occupying the space of the active pocket, which may affect the substrate specificity of the enzyme. The sequence alignment (Fig. 2) shows that the amino acid sequence of LDHs (corresponding to positions 12–17 of LDH0845) contains G-X-G-X-X-G, which is a typical NAD+ binding site highly conserved sequence (LI Fen, 2017). In addition, L149, D150, R153, and H177 are conserved in several hydroxy acid dehydrogenases, A220, Y221, I224, and T230 are slightly different. Therefore, the residues (R90A, Y221A, I224A, T230A) in the active pocket of LDH0845 were mutated into alanine separately, and it was expected to reduce the steric hindrance of the substrate-binding pocket and conduct kinetic characterization to evaluate the effect of each residue on catalysis (Supplementary Table 4). In short, Y221 is located in the active pocket and interferes with the entrance and binding of the substrate. And hydroxy acid dehydrogenases use different keto acids as natural substrates. It was found that Y221 is not conserved in the hydroxy acid dehydrogenases family through sequence alignment. Moreover, catalytic activity was improved by mutating tyrosine to alanine. Therefore, it can be considered to mutate Y221 to a hydrophobic residue or a smaller residue.
Fig. 2 Multiple sequence alignment of LDH0845 with LcLDH, BsLDH, L-HicDH, AHADH, SmHPPR. The residues in the blue frame are relatively conserved in several protein sequences, the residues in the red frame are completely conserved, and the residues in the black frame are related to the recognition and binding of the substrate and NADH.
Substrate specificity of LDH0845 and its mutants

The catalytic capacity of LDH0845 and Y221 mutants for different alpha-ketoacids were determined, and the results are shown in Fig. 3. The results show that all the 19 mutants maintained their catalytic capacity for pyruvate, and the specific activity of Y221K, Y221P and Y221W for pyruvate were 280.93 U/mg, 283.83 U/mg and 331.61 U/mg, respectively, which were 194%, 1.96% and 2.29% of the wild type. For 3,4-dihydroxyphenylpyruvate, the initial enzyme activity of the wild-type LDH0845 was only 12.30 U/mg, and the catalytic activity of the mutants Y221F, Y221S and Y221I were 339%, 232% and 229% of the wild type, respectively, showing the specific enzyme activity of 41.70 U/mg, 28.54 U/mg and 28.17 U/mg. In addition, the wild-type enzyme exhibited specific enzyme activity of 69.11 U/
mg for phenylpyruvate with a benzene ring on C3, and the mutants Y221F and Y221Q exhibited catalytic activity of 191.43 U/mg and 208.02 U/mg, which were 277% and 301% of the wild type, respectively. The wild-type LDH0845 had a specific enzyme activity of 16.08 U/mg for p-hydroxyphenylpyruvate, which also had large functional groups. Only Y221F and Y221P show improved catalytic activity, which were 186% and 168% of the wild-type LDH0845. However, when benzyol formic acid with benzene ring on C3 was used as the substrate, compared with the specific enzyme activity of wild-type LDH0845, none of the 19 mutants shows catalytic activity. In addition, the catalytic activities of mutants Y221A, Y221F, and Y221G on the smaller substrate glyoxylic acid were 64.98 U/mg, 40.21 U/mg, 45.85 U/mg, respectively, which are 265%, 1.64%, and 1.87% of the wild type. For 2-ketobutyric acid and 4-methyl-2-oxopentanoate, which also have long side chains, the effect of Y221 mutation on their catalytic activity was similar. The catalytic activity of mutant Y221K on 2-ketobutyric acid and 4-methyl-2-oxopentanoate were 29.93 U/mg and 67.43 U/mg respectively, were 187%, 180% of the wild-type LDH0845. The catalytic activities of mutant Y221E for 2-ketobutyric acid and 4-methyl-2-oxopentanoate were 37.58 U/mg and 101.14 U/mg, respectively, which were 226%, 270% of the wild-type. In addition, the specific activity of Y221A mutant for 4-methyl-2-oxopentanoate was 35.21 U/mg, which was only 94% of that of the wild-type enzyme, while specific activity of Y221F mutant for 4-methyl-2-oxopentanoate was 52.07 U/mg. The two mutants obviously had different effects on the catalytic activity. The wild enzyme exhibited specific enzyme activity of 21.17 U/mg for alpha-ketoglutaric acid. Y221A mutant retained 84% catalytic activity, and Y221V mutant was 194% of the original catalytic activity. The other mutants had no catalytic activity for alpha-ketoglutaric acid.

Temperature and pH characteristics of Y221F mutant

Since the mutant Y221F in this paper has the most significant increase in the activity of the substrate 3,4-dihydroxyphenylpyruvate, the relevant characteristics of it have been studied. The characterization research content includes the optimum temperature, optimum pH, temperature stability and pH stability. The SDS-PAGE analysis results of LDH0845 and Y221F mutants are shown in Fig. 4.

According to previous research of our team, the relative enzyme activity of wild-type LDH0845 at 20–35 °C was greater than 50%, which had a good catalytic effect [24]. It was found that both Y221F mutant and the wild-type enzyme show the highest enzyme activity at 25 °C through experimental data. At 20–25 °C, the relative enzyme activity of Y221F mutant was above 50%, but the enzyme activity decreased sharply as the temperature raised (Fig. 5a).

In addition, the thermal stability of Y221F mutants was investigated. After being incubated for 1.5 h at a temperature lower than 20 °C, this mutant still retained enzyme activity of more than 80%. However, after being incubated at temperature above 40 °C, this mutant only had residual enzyme activity of less than 20% (Fig. 5b). Y221F mutant and wild-type enzyme had the same optimal pH value, both of which were 6.0. When the value of pH was in the range of 5.0–6.0, Y221F mutant had residual enzyme activity more than 80%. However, when the value of pH was in the range of 4.0–5.0 and 6.0–9.0, this enzyme had a quite low enzyme activity. When the value of pH was in the range of 3.0 to 4.0, the enzyme basically lost its activity (Fig. 5c). In addition, the pH stability of Y221F mutant was also tested. It was found that the mutant had higher stability when the value of pH is between 4.5 and 6.0 through a large number of the experimental analysis of discovery. Furthermore, When the mutant was exposed to more acid and alkaline environment, the relative activity was significantly limited (Fig. 5d).
Enzyme activity and kinetic parameters of Y221F mutant

The kinetic parameters of Y221F mutant on several substrates are shown in Table 1. The $k_{cat}$ and $K_m$ values of Y221F mutant for 3,4-dihydroxyphenylpyruvate, 4-methyl-2-oxopentanoate and glyoxylate were determined and calculated according to the method described in 2.2.6. The results show that kinetic parameters of the mutants for 3,4-dihydroxyphenylpyruvate, 4-methyl-2-oxopentanoate and glyoxylate were different from those of the wild enzyme to varying degrees. The $k_{cat}$ values of Y221F mutant for 3,4-dihydroxyphenylpyruvate, 4-methyl-2-oxopentanoate and glyoxylate were 1.21 s$^{-1}$, 1.35 s$^{-1}$, and 0.72 s$^{-1}$, respectively, which were 4.2-, 1.5- and 1.3-times higher than those of the wild type. This mutation achieved a significant increase in catalytic efficiency. At the same time, the $K_m$ values of wild-type enzyme for the above three substrates were 11.37 mM, 3.52 mM and 3.69 mM. The values of Y221F mutant were 4.78 mM, 2.28 mM and 2.04 mM, respectively, showing an increase in affinity. In addition, according to the results of molecular docking, the binding energies of Y221F mutants were $-2.4$ kcal/mol, $-3.5$ kcal/mol, and $-4.9$ kcal/mol, respectively. The higher the absolute value of binding energy is, the more stable the binding is. The smaller the $K_m$ is, the greater the affinity is. Therefore, LDH0845 has stronger substrate specificity, lower binding energy and $K_m$ for glyoxylic acid.

Discussion

According to our previous research, LDH0845, which has the catalytic activity for alpha-ketocarboxylic acids, was screened. The enzyme has a high catalytic activity for
pyruvate, which is a natural substrate, and a weak catalytic activity for other alpha-keto carboxylic acids with large side chains. If the amino acids in the active pocket are polar amino acids, the hydrophobic interaction will be weaker. If the amino acids in the active pocket are amino acids with large side chains, the steric hindrance will be increased and the interaction between the enzyme and the substrate will interfere. Therefore, it can be considered to mutate the amino acids in the active capsule to hydrophobic amino acids or amino acids with smaller side chains. Ile224 is close to the side chain of the benzene ring of the substrate, occupying the space of the substrate pocket, which may affect the activity of the enzyme. A team had conducted site-directed saturation mutation on Ile229 of L.-LcLDH<sup>Q88R</sup>. The <i>k<sub>cat</sub></i> value of the double mutant L.-LcLDH<sup>Q88R/I229</sup> was 760 s<sup>−1</sup>, which was 1.4-fold increases than L.-LcLDH<sup>Q88R</sup> towards the substrates (Xue-Qing et al. 2018). However, the <i>k<sub>cat</sub></i> values of <i>BsLDH</i><sup>1240K</sup> and <i> BsLDH</i><sup>1240K</sup> were 48 s<sup>−1</sup> and 24 s<sup>−1</sup>, respectively, which were lower than 81 s<sup>−1</sup> of the wild-type enzyme [34]. In the early stage of this study, it was found through an alanine scan that the I224 position of LDH0845 did not have much effect on the substrate specificity and catalytic activity of the enzyme. The homology modeling and molecular docking results of LDH0845 were analyzed. It was found that Y221 is located in the substrate-binding pocket, which affects the size and shape of the active pocket and interfered with the entry of various substrates into the active center, thereby the catalytic activity of the enzyme on the substrate is influenced. According to the sequence alignment, subsequent molecular docking and site-directed mutagenesis studies, it was believed that Y221 was the key residue to increase the catalytic activity of LDH0845 towards 2-oxo acids with larger side chains compared to a natural substrate (Fig. 2).

L.-HiCDH is an enzyme with the ability to reduce 2-keto-carboxylic acid stereospecifically, derived from <i>Lactobacillus</i> fusion [36]. The <i>k<sub>cat</sub></i> value of the wild-type enzyme is 7300 s<sup>−1</sup> for phenylpyruvate [11]. The Y237 of AHADH is replaced with Gly, and the <i>k<sub>cat</sub></i> value of the mutant for phenylpyruvate increases from 352 s<sup>−1</sup> of the wild-type enzyme to 488 s<sup>−1</sup> [27, 41]. ESPript 3.0 was used for sequence alignment of LDH0845, L.-HiCDH and AHADH [33]. It was found that the three types of dehydrogenases are conserved in the residues directly involved in the binding of substrate C<sub>1</sub> carboxylate and catalysis, such as His177, Asp150, and Arg153 (Fig. 2). But the position 221 is different: this is a polar amino acid Tyr in LDHs and AHADH; it is a non-polar amino acid Phe in L.-HiCDH, which has larger polar interaction with the substrate. The F236 of L.-HiCDH (corresponding to Y221 of LDH0845) was mutated, F236V mutant and F236S mutant were obtained. The <i>k<sub>cat</sub></i> value of F236V with hydrophobic amino acid toward phenylpyruvate increases to 15000 s<sup>−1</sup>, and the <i>k<sub>cat</sub></i> value of F236S with hydrophilic amino acid toward phenylpyruvate decreases to 6400 s<sup>−1</sup>. The intermolecular contact of wild-type LDH0845, Y221F mutant and Y221Q mutant with phenylpyruvate were analyzed to determine the effect of the mutation on the substrate specificity of the enzyme (Fig. 6). First of all, the mutations of Y221F and Y221Q changed the hydrophilic amino acid Tyr at position 221 to the hydrophobic amino acid Phe and Gln, which has better hydrophobic interaction than the inferior hydrophilic-hydrophobic contact in the wild-type enzyme. In addition, both mutations make the active pockets significantly larger, the steric hindrance was reduced, and the binding of the substrate is more favorable.

<i>SmHPPR</i>, hydroxyphenylpyruvate reductase from <i>S. miltiorrhiza</i>, had <i>k<sub>cat</sub></i> value of 85 s<sup>−1</sup> for 3,4-dihydroxyphenylpyruvate [41, 43]. The 221 position of the enzyme was small non-polar hydrophobic amino acid Val. Therefore, the enzyme had a larger substrate pocket, which was more conducive to turnover of 3,4-dihydroxyphenylpyruvate. For 3,4-dihydroxyphenylpyruvate, the initial enzyme activity of the wild-type LDH0845 was only 12.30 U/mg, and the catalytic activity of the mutants Y221F, Y221S and Y221I were increased by 3.39-, 2.32- and 2.29- times, respectively, showing the specific enzyme activity of 41.70 U/mg, 28.54 U/mg and 28.17 U/mg. In order to determine

![Fig. 6](image)

**Fig. 6** The docking diagram of wild-type LDH0845 (a), Y221F mutant (b) and Y221Q (c) mutant with phenylpyruvate. The active pocket of the enzyme is displayed in the form of the surface, the hydrogen bonds are shown by yellow dashed lines, NADH is shown by bright yellow and phenylpyruvate is shown by rainbow colors.
the effect of the mutation on the catalytic for 3,4-dihydroxyphenylpyruvate, wild-type LDH0845 and Y221F mutant, Y221S mutant, Y221I mutant were docked with substrate respectively. The docking results show that the unfavorable hydrophilic contact and steric hindrance of Tyr221 with a substituent of 3,4-dihydroxyphenylpyruvate made the entire substrate rotate along the axis of the C1–C2 bond (Fig. 7). Y221F mutant and Y221I mutant had a hydrophobic amino acid at residue 221, which had a greater hydrophobic interaction and made substrate-binding more stable. Residue 221 of other mutants had a smaller side chain than the wild-type enzyme, these mutants had larger active pockets and smaller steric hindrance. It was easier for them to accommodate larger substrates.

The Y237 of AHADH was replaced with the smallest amino acid Gly to achieve an increase in $k_{\text{cat}}$ value for $p$-hydroxyphenylpyruvate from 447 to 669 s$^{-1}$ [27, 41]. $Sm$HPPR had $k_{\text{cat}}$ value of 24 s$^{-1}$ for $p$-hydroxyphenylpyruvate [43]. The docking results of wild-type, Y221F mutant and Y221P mutant with $p$-hydroxyphenylpyruvate show that phenylalanine and proline had smaller amino acid side chains (Fig. 8). Therefore, compared with the wild type, the substrate active pocket of mutants became larger, and changes of space and shape were more favorable for entry of substrate and binding with the enzyme. Secondly, polar
hydrophilic amino acid Tyr was replaced by non-polar hydrophobic amino acids Pro and Phe, the hydrophobic interaction was increased, and the catalytic activity was enhanced.

The Val108 in Pseudomonas stutzeri SDML-LDH was replaced with Ala, and its catalytic efficiency for l-mandelic acid was more than 50 times that of the wild-type enzyme [19]. However, when Mandelate dehydrogenase from Pseudomonas putida catalyzed benzoyl formic acid, Arg165 and Arg258 formed hydrogen bonds with two oxygen atoms in the carboxyl group, and His255 and Tyr131 interacted with the oxygen atom of C2. So that benzoyl formic acid was kept in a relatively favorable catalytic position [9, 19]. To verify that Tyr221 plays an important role in catalysis of benzoyl formic acid, LDH0845 and Y221F mutant, which were highly active toward larger substrates, were respectively molecularly docked with benzoyl formic acid. The results are shown in Fig. 9, benzoyl formic acid was catalyzed by hydrogen bonds formed with Arg153 and His177 of wild-type LDH0845. The docking results of Y221F mutant with benzoyl formic acid show that this mutation realized expansion of the substrate pocket, but the distance between benzoyl formic acid and the surrounding residues became larger, and the hydrogen bond interaction between benzoyl formic acid and His177 disappeared, and only one hydrogen bond was formed with the guanidine group of Arg. Moreover, the benzene ring of benzoyl formic acid was farther away from the benzene ring of F221 than that of Y221, which led to the weakening of π–π stacking. These may be the reason why Y221F mutant lost the catalytic activity towards benzoyl formic acid.

l-lactate dehydrogenase from potato tubers could catalyze glyoxylate with specific enzyme activity of 18.5 µmol/min/mg (D D, 1972). In addition, the specific activity of l-LDH from Cupriavidus necator towards pyruvate was 824.6 µmol/min/mg, and the catalytic activity toward 4-methyl-2-oxopentanoate was 39% of that toward pyruvate (STEINBÜCHEL and Schlegel, 1983). Lactate dehydrogenase derived from Plasmodium falciparum also had weak activity toward 4-methyl-2-oxopentanoate [18]. The amino acids of Y221 at the corresponding positions of the above three enzymes are Tyr, Lys, and Leu. Therefore, large aromatic amino acids would not interfere with the binding and catalysis of the small substrate like glyoxylate. However, when 4-methyl-2-oxopentanoic acid was used as a substrate, smaller amino acid residues may be a better choice for the active pocket, such as positively charged lysine and aliphatic hydrophobic amino acid leucine.

The specific activity of Y221A mutant for 4-methyl-2-oxovaleric acid is 35.21 U/mg, which is only 94% of the wild-type enzyme, while the specific activity of Y221F mutant for 4-methyl-2-oxovaleric acid is 52.07 U/mg, which is 139% of the wild-type enzyme. The specific activity of Y221A mutant and Y221F mutant for glyoxylate is 64.98 U/mg and 40.21 U/mg, respectively, which is 265% and 164% of the wild-type enzyme. LDH0845, Y221F mutant and Y221A mutant were molecularly docked with 4-methyl-2-oxopentanoate and glyoxylate, respectively. The results are shown in Fig. 10, C2 of glyoxylate and 4-methyl-2-oxopentanoate were separately connected to a hydrogen atom and a short-chain with five carbon atoms. Both wild enzyme and Y221F mutant can catalyze these two substrates through Arg153 and His177, and the aromatic amino acid at position 221 would not have obvious negative effects on the binding and catalysis of the substrate. Y221A mutant had a larger active pocket, which made it easier for glyoxylate and 4-methyl-2-oxopentanoate to enter into the active center, so the catalytic efficiency of Y221A mutant was higher theoretically. However, the docking results show that the distance between the substrate and the amino acid residues and NADH became larger due to mutations. Moreover, the effect of interaction between aromatic amino acid residues on the thermal stability and structural stability of proteins cannot be ignored [6, 21]. Therefore, the mutation of Y221A may weaken the structural stability of complex formed by the combination of enzyme and 4-methyl-2-oxopentanoate and resulted in a decrease in catalytic efficiency. However, the specific catalytic mechanism needs further verification.

**Fig. 9** The docking diagram of wild-type LDH0845 (a), Y221F mutant (b) with benzoyl formic acid. The active pocket of the enzyme is displayed in the form of the surface, NADH is displayed in yellow, benzoyl formic acid is displayed in rainbow colors, the hydrogen bonds are shown by yellow dashed lines.
Conclusion

In summary, this research changed the substrate spectrum of LDH0845 and obtained mutants with catalytic activity for some 2-oxoketo acids with larger side chains through rational design, site-directed mutagenesis and subsequent experimental screening. Among them, the catalytic activity of mutant Y221F on 3,4-dihydroxyphenylpyruvate, p-hydroxyphenylpyruvate, 4-methyl-2-oxopentanoate are 41.70 U/mg, 29.91 U/mg, 101.14 U/mg, corresponding to 3.39-, 1.86-, and 2.70-fold of the wild type, respectively. The mutants as better bio-catalysis could produce aromatic lactic acids, which have important industrial application potential.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Fig. 10 The docking diagram of wild-type LDH0845 (a, d), Y221F mutant (b, e) and Y221A mutant (c, f) with 4-methyl-2-oxopentanoate and glyoxylate. The active pocket of the enzyme is displayed in the form of the surface, the hydrogen bonds are shown by yellow dashed lines, NADH is shown by bright yellow, 4-methyl-2-oxopentanoate and glyoxylic acid are shown by rainbow colors.
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