Modified Catalytic Performance of *Lactobacillus Fermentum* L-lactate Dehydrogenase by Rational Design

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

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

28 **Keywords:** L-lactate dehydrogenases, single-point mutation, substrate specificity, rational design

29

30 1. Introduction

31 L-lactate dehydrogenase (L-LDH, EC 1.1.1.27), an oxidoreductase of NAD^+ dependent L-specific hydroxy acid
32 dehydrogenases family, exists in human beings, animals, plants and microorganisms in the form of tetramer (LDH-M4,
33 LDH-M3H, LDH-M2H2, LDH-M3H, LDH-H4)(Caltrider and Lehman, 1975). L-hydroxy acid dehydrogenases are kinds
34 of homologous protein that use NAD(P)H as cofactor to realize the stereospecific conversion of latent alpha-keto acids
35 and chiral alpha-hydroxy acids(Han et al., 2014). The enzymes in the alpha hydroxy acid dehydrogenases family can be
36 classified and named according to their optimal substrates. In this family, several enzymes that have been studied
37 frequently include lactate dehydrogenase, malate dehydrogenase(Grant, 1989), glyoxylate reductase, 2-hydroxyglutarate
38 dehydrogenase (Booth et al., 2006), 2-hydroxyisocaproate dehydrogenase (Niefind et al., 1995) and mandelate
39 dehydrogenase and a long chain alpha-hydroxy acid oxidase (Hao et al., 2007). Generally, LDHs could be obtained from
40 a variety of sources and have a higher affinity for pyruvate as a natural substrate. However, some known wild-type LDHs
41 have their own substrates specificity and have high catalytic efficiency for other substrates with different side chains. For
42 example, wild-type *Bs*LDH has the catalytic activity for 4-methyl-2-oxopentanoate with long side chains and
43 phenylpyruvate with benzene ring(Kallwass et al., 1992); L-LDHs derived from *Clonorchis sinensis* and
44 *Thermoanaerobacter ethanolicus* JW200 has different catalytic efficiency for multiple substrates including pyruvic acid,
45 2-ketobutanoic acid, oxaloacetic acid, ketoglutarate, phenylpyruvate and so on(Yang et al., 2006; Zhou and Shao, 2010).
46 In addition, there are some other dehydrogenases that have better catalytic activity for larger side chain keto acids. For
47 example, L-HicDH derived from *Lactobacillus confusus* has the ability to sterically reduce 2-ketocarboxylic acid (Feil et
48 al., 1997); AHADH derived from *Trypanosoma cruzi* has the value of k_{cat} for p-hydroxyphenylpyruvate is 447 s^{-1} (Vernal
49 et al., 2002) ; *Sm*HPPR, a hydroxyphenylpyruvate reductase from *S. miltiorrhiza*, has the value of k_{cat} for 3,4-
50 dihydroxyphenylpyruvate is 85 s^{-1} (Wang et al., 2017). Therefore, we can change the substrate specificity of enzymes
51 through protein engineering to increase their affinity for different 2-oxo acids. Through the structural comparison between

different enzymes and rational design, the active pocket of LDHs was changed to suit target substrates with different size substituents, so that it had higher catalytic activity for various alpha - ketocarboxylates. Ultimately, the different alpha - hydroxy acids obtained were widely used in industrial production(Tian et al., 2006; Wagner and Maierhofer, 1978; Xue-dan et al., 2008; Yevglevskis et al., 2014).

A lactate dehydrogenase gene, LF-L-LDH0845 (GenBank accession MG581696), was cloned from *Lactobacillus fermentum* JN248 in our laboratory. Furthermore, 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. Values of K_m , k_{cat} , and k_{cat} / K_m of LF-L-LDH0845 for 3,4-dihydroxyphenylpyruvate is 11.37 mM, 0.2931 s⁻¹, and 0.0258 mM⁻¹·s⁻¹, respectively. LF-L-LDH0845 has an approximate molecular weight of 33.65 kDa(Lu et al., 2018). 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.

2. Materials and Methods

2.1 Materials

2.1.1 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.

2.1.2 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 (Bai et al., 2014). 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), QuickCut™ 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.

2.2 Methods

2.2.1 Homology modelling and molecular docking of LDH0845

The crystal structure of L-lactate dehydrogenase (*Lp*LDH, PDB:[1EZA](#)) 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 (Bertoni et al., 2017; Bienert et al., 2016; Guex et al., 2009; Studer et al., 2019; Waterhouse et al., 2018), and then SAVES v6.0 (<https://saves.mbi.ucla.edu/>) was used to evaluate the modeling results (Bowie et al., 1991; Lüthy et al., 1992). 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*, *CMLDH*, *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://esprict.ibcp.fr/ESPript/ESPript/>).

2.2.2 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 *Lactobacillus fermentum* JN248. The plasmid pCold II was digested with Restriction endonucleases BamHI and Sac I, and then purified using a purification kit. Afterwards, target gene was ligated to pCold II with ABclonal MultiF Seamless Assembly ligase to construct a recombinant plasmid.

2.2.3 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 (Reetz and Carballeira, 2007). 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.

114 2.2.4 Expression and purification of L-LF-LDH0845

115 *E. coli* BL21 containing wild-type lactate dehydrogenase or its mutants were inoculated into Luria-Bertani
116 medium with ampicillin (100 mg/mL), and cultured overnight at 37 °C, 220 rpm. The culture was inoculated into 50
117 mL Luria-Bertani medium containing ampicillin (100 mg/mL) at a volume ratio of 2%. It was shaken in a shaker at
118 37 °C and cultivated until the value of OD₆₀₀ was approximately 0.6 - 0.8. It was stably cultured at 15 °C for 30 min
119 for pre-cooling. Later, IPTG was added to the final concentration of 0.4 mM, and then it was incubated for 24 hours
120 at 15 °C, 200 rpm (Quan et al., 2018). The bacterial solutions were centrifuged at 4 °C, 8000×g for 10 min, and the
121 supernatants were discarded to collect the cells. The precipitates were suspended in phosphate buffer (pH 6.0) and
122 sonicated (The power is 200W, the time is 20 min, Ultrasound is turned on for 2s, Ultrasound is turned off for 2s) to
123 break the cells, and then centrifuged again to remove cell debris, and the supernatants were crude enzyme solutions.

124 The crude enzyme solutions were filtered through 0.22 µm membrane and then separated and purified on the
125 AKTA Avant chromatography system using HisTrap™ HP 5 mL column. The Ni²⁺ affinity column was equilibrated
126 with binding buffer (20mM sodium phosphate, 0.5M NaCl, 5 mM imidazole, pH7.4), and then His-tagged
127 recombinase was treated by linear elution with washing buffer (20mM sodium phosphate, 0.5M NaCl, 500 mM
128 imidazole, pH7.4). The eluted proteins were desalted to remove imidazole through a 5 mL HisTrap desalting column.
129 The purified recombinant enzymes were analyzed on 12% SDS-PAGE and the concentrations of purified proteins
130 were measured using the BCA protein concentration determination kit. (Prasad et al., 2012; Zhaojuan et al., 2011).

131 2.2.5 Activity assay and substrate specificity of L-LF-LDH0845 mutant

132 The enzyme activity of LDH0845 was determined based on the change in absorbance of NADH at 340 nm (Pan
133 et al., 2003). The absorbance was measured using a spectrometer (Prismatic Technology 759S UV-Vis
134 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-oxybutyric 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 3mL 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 15min, 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 μ mol 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.

2.2.6 Characteristics of Y221F mutant

The reaction system composed of disodium hydrogen phosphate-sodium dihydrogen phosphate (pH 6.0) was placed at different temperatures (10 °C-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 1h, 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

156 is the same as 2.2.5. The enzyme activity of the untreated enzyme solutions was defined as 100%, and the enzyme activity
157 of the enzyme solutions treated by other conditions was the relative value. Substrates of different concentrations (0.25-7
158 mM) were added to the reaction system and placed in the optimal conditions (25 °C, pH 6.0) for 15 min. The enzyme
159 solution with a protein concentration of 0.56 mg/mL was appropriately diluted and added to it, and the absorbance
160 measurement and enzyme activity calculation were performed according to the methods mentioned in 2.2.5. Finally, the
161 kinetic parameter data was calculated by the nonlinear fitting (the category is Growth/sigmoidal, the function is Hill, n=1)
162 of Origin2010 software.

163 **3 results**

164 **3.1 Homology modelling and mutation site design of LDH0845**

165 After the modeling result is verified (Supplementary Note 2), NADH and natural substrate pyruvate were docked
166 with LDH0845. The docking results are shown in Figure 1. Several important residues around the substrate active
167 pocket were the same as those of *Lp*LDH, including residues Leu149, Arg153, and His177, which are involved in
168 catalysis and substrate binding, as well as the residues A220, Y221, I224 and T230. Among them, R153 forms a
169 dihydrogen bond with the carboxyl group of pyruvate through the guanidine group to facilitate the correct binding of
170 the substrate at the catalytic site, H177 acts as a proton donor/acceptor to transfer hydrogen atoms in redox reactions
171 (Hayao et al., 2014; Matsuzawa et al., 1988; Schroeder et al., 1988). Y221 is located in the active pocket and interferes
172 with the entry and binding of the substrate. However, Y221 is relatively conservative in LDHs and is not fixed in other
173 enzymes of the same family. In addition, Ile224 is close to the side chain of the substrate, occupying the space of the
174 active pocket, which may affect the substrate specificity of the enzyme. The sequence alignment (Figure 2) shows that
175 the amino acid sequence of LDHs (corresponding to positions 12-17 of LDH0845) contain G-X-G-X-X-G, which is a
176 typical NAD⁺ binding site highly conserved sequence (Li Fen, 2017). In addition, L149, D150, R153, and H177 are

177 conserved in several hydroxy acid dehydrogenases, A220, Y221, I224, and T230 are slightly different. Therefore, the
178 residues (R90A, Y221A, I224A, T230A) in the active pocket of LDH0845 were mutated into alanine separately, and it
179 was expected to reduce the steric hindrance of the substrate-binding pocket and conduct kinetic characterization to
180 evaluate the effect of each residue on catalysis (Supplementary Table 4). Finally, it can be considered to mutate Y221
181 to a hydrophobic residue or a smaller residue.

182

183 **Figure 1**

184 **Figure 2**

185 **3.2 Substrate specificity of LDH0845 and its mutants**

186 The catalytic capacity of LDH0845 and Y221 mutants for different alpha-ketoacids were determined, and the results
187 are shown in Fig 3. The results show that all the 19 mutants maintained their catalytic capacity for pyruvate, and the
188 specific activity of Y221K, Y221P and Y221W for pyruvate were 280.93 U/mg, 283.83 U/mg and 331.61U/mg,
189 respectively, which were 194%, 1.96% and 2.29% of the wild type. For 3,4-dihydroxyphenylpyruvate, the initial enzyme
190 activity of the wild-type LDH0845 was only 12.30 U/mg, and the catalytic activity of the mutants Y221F, Y221S and
191 Y221I were 339%, 232% and 229% of the wild type, respectively, showing the specific enzyme activity of 41.70 U/mg,
192 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
193 phenylpyruvate with a benzene ring on C3, and the mutants Y221F and Y221Q exhibited catalytic activity of 191.43
194 U/mg and 208.02 U/mg, which were 277% and 301% of the wild type, respectively. The wild-type LDH0845 had a specific
195 enzyme activity of 16.08 U/mg for p-hydroxyphenylpyruvate, which also had large functional groups. Only Y221F and
196 Y221P show improved catalytic activity, which were 186% and 168% of the wild-type LDH0845. However, when benzoyl
197 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% the original catalytic activity. The other mutants had no catalytic activity for alpha-ketoglutaric acid.

Figure 3

3.3 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.

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 (Lu et al., 2018). 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 temperature raised

(Figure 4a). In addition, the thermal stability of Y221F mutants was investigated. After being incubated for 1.5h at 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% (Figure 4b).

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 (Figure 4c). 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 4.5-6.0 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 (Figure 4d).

Figure 4

3.4 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 1.21s⁻¹, 1.35s⁻¹, and 0.72s⁻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 4.78mM, 2.28mM and 2.04mM, respectively, showing an increase in affinity.

Table 1

4. 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 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 the large side chain, the steric hindrance will be increase 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^{Q88R}. The k_{cat} value of the double mutant L-LcLDH^{Q88R/I229Q} was 760 s⁻¹, which was 1.4 - folds increases than L-LcLDH^{Q88R} towards the substrates (Xue-Qing et al., 2018). However, the k_{cat} values of B_sLDH^{I240R} and B_sLDH^{I240K} were 48 s⁻¹ and 24 s⁻¹, respectively, which were lower than 81 s⁻¹ of the wild-type enzyme (Roman et al., 1995). In the early stage of this study, it was found through 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 natural substrate (Fig 2).

L-HicDH is an enzyme with the ability to reduce 2-ketocarboxylic acid stereospecifically, derived from *Lactobacillus fusion* (Schütte et al., 1984). The k_{cat} value of the wild-type enzyme is 7300 s^{-1} for phenylpyruvate (Feil et al., 1997). The Y237 of AHADH is replaced with Gly, and the k_{cat} value of the mutant for phenylpyruvate increases from 352 s^{-1} of the wild-type enzyme to 488 s^{-1} (Montemartini et al., 1994; Vernal et al., 2002). ESPript 3.0 was used for sequence alignment of LDH0845, L-HicDH and AHADH (Robert and Gouet, 2014). It was found that the three types of dehydrogenases are conserved in the residues directly involved in binding of substrate C₁ carboxylate and catalysis, such as His177, Asp150, and Arg153 (Figure 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 k_{cat} value of F236V with hydrophobic amino acid toward phenylpyruvate increases to 15000 s^{-1} , and the k_{cat} value of F236S with hydrophilic amino acid toward phenylpyruvate decreases to 6400 s^{-1} . 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 (Figure 5). 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 reduces, and the binding of the substrate is more favorable.

Figure 5

SmHPPR, hydroxyphenylpyruvate reductase from *S. miltiorrhiza*, had k_{cat} value of 85 s^{-1} for 3,4-dihydroxyphenylpyruvate (Vernal et al., 2002; Wang et al., 2017). The 221 position of 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 41.70U/mg, 28.54U/mg and 28.17 U/mg. In order to determine 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 (Figure 6). 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.

Figure 6

The Y237 of AHADH was replaced with the smallest amino acid Gly to achieve an increase in k_{cat} value for p-hydroxyphenylpyruvate from 447 s^{-1} to 669 s^{-1} (Montemartini et al., 1994; Vernal et al., 2002). *SmHPPR* had k_{cat} value of 24 s^{-1} for p-hydroxyphenylpyruvate (Wang et al., 2017). 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 (Figure 7). 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 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.

Figure 7

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 (Jiang et al., 2012). 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 (Dewanti and Mitra, 2003; Jiang et al., 2012). 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 Figure 8, 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.

Figure 8

L-lactate dehydrogenase from *potato tubers* could catalyze glyoxylate with specific enzyme activity of 18.5 $\mu\text{mol}/\text{min}/\text{mg}$ (D D, 1972). In addition, the specific activity of L-LDH from *Cupriavidus necator* towards pyruvate was 824.6 $\mu\text{mol}/\text{min}/\text{mg}$, 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-oxopentate (Hewitt et al., 1997). 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.

Figure 9

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 64.98U/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 Figure 9, 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 active center, so the catalytic efficiency of Y221A mutant was higher theoretically. However, the effect of interaction between aromatic amino acid residues on the thermal stability and structural stability of proteins cannot be ignored(Burley et al., 1985; Kannan and Vishveshwara, 2000). 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.

Conclusion

In summary, this research carried out the prediction and simulation of protein structure through homology modelling, and initially designed the modification of protein through molecular docking. Then, further screening was carried out through experiments, and the mutants with catalytic activity to some 2-oxo-keto acids with larger side chains were initially obtained. Among them, the catalytic activity of mutant Y221F on 3,4-dihydroxyphenylpyruvate, p-

347 hydroxyphenylpyruvate, 4-methyl-2-oxopentanoate is significantly improved, and the reduction products have a broad
348 vision of application. Despite the above achievements, there are still some issues worthy of further discussion. On this
349 basis, it is feasible to carry out combined mutations to obtain better mutants, and it is necessary to explore the existence
350 of allosteric effects.

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466

467 **Ethics declarations**

468 **1.Conflict of interest**

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

470 **2.Ethical approval**

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

472 **3.Informed consent**

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

474

475

476 **Figure legends**

477 **Figure 1.** The docking result of pyruvate (red) and A chain of wild-type LDH0845 (dark green). The blue frame is a
478 partially enlarged view of the binding part of the pyruvate and the enzyme, which contains pyruvate and several
479 important amino acids (deep-teal cyan) near the active pocket.

480 **Figure 2.** Multiple sequence alignment of LDH0845 with LcLDH, BsLDH, L-HicDH, AHADH, *SmHPPR*. The
481 residues in the blue frame are relatively conserved in several protein sequences, the residues in the red frame are
482 completely conserved, and the residues in the black frame are related to the recognition and binding of the substrate
483 and NADH.

484 **Figure 3.** Relative enzyme activity of Y221 mutant. The corresponding relationship between the substrates and the
485 serial number in the figure in the figure is as follows. (1: pyruvate; 2: 3,4-dihydroxyphenylpyruvate; 3: p-
486 hydroxyphenylpyruvate; 4: 3-methyl-2-oxybutyric acid; 5: alpha-ketoglutaric acid; 6: phenylpyruvate; 7: oxaloacetic
487 acid; 8: 2-ketobutyric acid; 9: 4-methyl-2-oxopentanoate; 10: benzoyl formic acid; 11: glyoxylate.)

488 **Figure 4.** pH and temperature profiles of Y221F mutant. (a) Effect of temperature on
489 enzyme activity. (b) Thermostability of Y221F mutant. (c) Effect of pH on enzyme activity.
490 (d) pH stability of Y221F mutant.

491 **Figure 5.** The docking diagram of wild-type LDH0845 (a), Y221F mutant (b) and Y221Q (c) mutant with phenylpyruvate.
492 The active pocket of the enzyme is displayed in the form of the surface (milk white), the hydrogen bonds are shown by
493 yellow dashed lines.

494 **Figure 6.** The docking diagram of wild-type LDH0845 (a), Y221F mutant (b), Y221S mutant (c) and Y221I (d) mutant
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496 3,4-dihydroxyphenylpyruvate is displayed in rainbow colors composed of orange, red, blue and white, the hydrogen
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501 are shown by yellow dashed lines.

502 **Figure 8.** The docking diagram of wild-type LDH0845, Y221F mutant with benzoyl formic acid. The active pocket of
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505 **Figure 9.** The docking diagram of wild-type LDH0845 (a, d), Y221F mutant (b, e) and Y221A mutant (c, f) with 4-
506 methyl-2-oxopentanoate and glyoxylate. The active pocket of the enzyme is displayed in the form of the surface (milk
507 white), the hydrogen bonds are shown by yellow dashed lines, 4-methyl-2-oxopentanoate and glyoxylate are displayed in
508 rainbow colors composed of orange, red and white.

509

510 **Table 1.** Kinetic parameters of Y221F mutant for various substrates.

Substrate	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat} / K_m (mM ⁻¹ ·s ⁻¹)
3,4-dihydroxyphenylpyruvate	4.78±0.27	1.21±0.08	0.25
4-methyl-2-oxopentanoate	2.28±0.37	1.35±0.05	0.59
glyoxylate	2.04±0.11	0.72±0.01	0.35

517

518

519 **Figure 1.** The docking result of pyruvate (red) and A chain of wild-type LDH0845 (dark green). The blue frame is a

520 partially enlarged view of the binding part of the pyruvate and the enzyme, which contains pyruvate and several

521 important amino acids (deep-teal cyan) near the active pocket.

522

	1	10	20	30
LDH0845	MSKTHQ	KVVLNSDGTDS	PSYAFAMV	QQGLAQ
LcLDH	MASITDKDHQ	KVILVGDGAVC	SSYAYAMVL	QGIAQ
BsLDH	MKNNGGA	RVVVGAGFV	CASYVFA	LMNQIAD
CNLDH	MHQPK	IVVVGAGLV	GSALFA	AVAIIPSA
PTLDH	MSSSSFLSLDGLDLNQVF	FKSISNADPPSQT	NHHTKIS	SVIGVGNVCM
HicDH	AR	KIGLIGLGN	VCAAVAH	GLIAQGVAD
AHADH	MFFEACAKVVVSG	AGQVGYAL	LF	LIAGGRMLGPNQHL
SmHPPR	MEAI	GVLM MCP	MS	TYLBOELDRFRKLF

	40	50	60	70	80
LDH0845	EF	AIIDLNKKH	..TEGDALD	LEDATPFT	APKL
LcLDH	EIG	IVDIFKDK	..TKGDA	IDLSNALPFT	SPKK
BsLDH	EIV	LIDANESK	..AIGDAM	D FNHGKV	FAPK
CNLDH	EV	VIDVARIR	..AEGQALD	LAHSAAPW	GNNRF
PTLDH	EL	ALVDVNSDK	..LRGEMLD	LQHAAAF	LPRTKI
HicDH	DY	VIDANEAK	..VKADQID	FQDAMAN	LEAHGN
AHADH	QL	NLLDIEPAMKC	LEGIR	AE	LMDCAF
SmHPPR	DF	LALQAESIR	..AVV	GN	NA

	90	100	110	120	130	140
LDH0845	K	P	GETRDL	DLV	DK	NLKI
LcLDH	K	P	GETRDL	DLV	NK	NLKI
BsLDH	K	P	GETRDL	DLV	DK	NIAI
CNLDH	K	P	GETRDL	DLA	KTNAG	IAAE
PTLDH	N	P	GESRLN	LLQ	RNLAM	YKSI
HicDH	N	P	TGERFA	ELK	F	TSSM
AHADH	K	P	GTIR	DL	LQKNA	AI
SmHPPR	T	P	VIT	DL	VA	DLA

	150	160	170	180	190	200
LDH0845	V	G	S	G	T	S
LcLDH	V	G	S	G	T	S
BsLDH	I	G	S	G	T	I
CNLDH	I	S	T	G	T	S
PTLDH	I	G	S	G	T	S
HicDH	I	G	T	G	T	S
AHADH	V	S	A	L	T	R
SmHPPR	I	G	L	A	V	A

	210	220	230	240	250
LDH0845	KG	ISKDD	LYK	LEDD	VRNKA
LcLDH	HP	EIKED	KLVK	MFED	VRDA
BsLDH	KG	E	EAKQDLER	IFVN	VRDA
CNLDH	TG	KELGPAS	RDL	ILRS	VHEA
PTLDH	QQ	TAF	EKDTLEK	IKHQ	VVSAYE
HicDH	GD		IDLAA	IEEE	ARKG
AHADH	DA		YFDGE	FMTT	VQQR
SmHPPR	II		NRE	VIDA	LGP

	260	270	280	290	300	310
LDH0845	A	P	M	S	G	E
LcLDH	V	Y	M	D	G	Q
BsLDH	A	Y	L	D	G	L
CNLDH	I	V	H	P	E	V
PTLDH	I	L	V	K	G	F
HicDH	N	R	R	D	M	G
AHADH	V	Y	S	D	G	N
SmHPPR	E	V	P	E	K	L

LDH0845	IQG....
LcLDH	NDIETRQ
BsLDH	
CNLDH	R.....
PTLDH	
HicDH	L.....
AHADH	AL.....
SmHPPR	

524

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526 residues in the blue frame are relatively conserved in several protein sequences, the residues in the red frame are
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535

536

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540

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546

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550 bonds are shown by yellow dashed lines.

551

Figure 7. The docking diagram of wild-type LDH0845 (a), Y221F mutant (b), Y221P mutant (c) with p-hydroxyphenylpyruvate. The active pocket of the enzyme is displayed in the form of the surface (milk white), p-hydroxyphenylpyruvate is displayed in rainbow colors composed of orange, red, blue and white, the hydrogen bonds are shown by yellow dashed lines.

558

559 **Figure 8.** The docking diagram of wild-type LDH0845, Y221F mutant with benzoyl formic acid. The active pocket of
 560 the enzyme is displayed in the form of the surface (milk white), benzoyl formic acid is displayed in rainbow colors
 561 composed of orange, red, blue and white, the hydrogen bonds are shown by yellow dashed lines.

562

Figure 9. 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 (milk white), the hydrogen bonds are shown by yellow dashed lines, 4-methyl-2-oxopentanoate and glyoxylate are displayed in rainbow colors composed of orange, red and white.

Supplementary Files

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