Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

Cloning and characterization of an oxiranedicarboxylate hydrolase from *Labrys* sp. WH-1*#

Wen-na BAO[1,2,3], Zi-sheng LUO[†‡1], Shi-wang LIU[2,3], Yuan-feng WU[2,3], Pei-lian WEI[2,3], Gong-nian XIAO[2,3], Yong LIU[2,3]

[1]*College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China*
[2]*School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou 310023, China*
[3]*Zhejiang Provincial Key Laboratory for Chemical and Biological Processing Technology of Farm Products, Hangzhou 310023, China*
[†]E-mail: luozisheng@zju.edu.cn

Received July 8, 2019; Revision accepted Sept. 5, 2019; Crosschecked Oct. 8, 2019

Abstract: Objective: This study aimed to clone and characterize the oxiranedicarboxylate hydrolase (ORCH) from *Labrys* sp. WH-1. Methods: Purification by column chromatography, characterization of enzymatic properties, gene cloning by protein terminal sequencing and polymerase chain reaction (PCR), and sequence analysis by secondary structure prediction and multiple sequence alignment were performed. Results: The ORCH from *Labrys* sp. WH-1 was purified 26-fold with a yield of 12.7%. It is a monomer with an isoelectric point (p*I*) of 8.57 and molecular mass of 30.2 kDa. It was stable up to 55 °C with temperature at which the activity of the enzyme decreased by 50% in 15 min (T_{50}^{15}) of 61 °C and the half-life at 50 °C ($t_{1/2,\ 50\ °C}$) of 51 min and was also stable from pH 4 to 10, with maximum activity at 55 °C and pH 8.5. It is a metal-independent enzyme and strongly inhibited by Cu^{2+}, Ag^{+}, and anionic surfactants. Its kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were 18.7 mmol/L, 222.3 s^{-1}, and 11.9 mmol/(L·s), respectively. The *ORCH* gene, which contained an open reading frame (ORF) of 825 bp encoding 274 amino acid residues, was overexpressed in *Escherichia coli* and the enzyme activity was 33 times higher than that of the wild strain. Conclusions: The catalytic efficiency and thermal stability of the ORCH from *Labrys* sp. WH-1 were the best among the reported ORCHs, and it provides an alternative catalyst for preparation of L(+)-2,3-dihydrobutanedioic acid.

Key words: Oxiranedicarboxylate hydrolase (ORCH); L(+)-2,3-Dihydrobutanedioic acid; Characterization; Cloning
https://doi.org/10.1631/jzus.B1900392 **CLC number:** Q556

1 Introduction

Oxiranedicarboxylate hydrolase (ORCH) belongs to the epoxide hydrolase (EC 3.3.2.3) family and can asymmetrically hydrolyze oxiranedicarboxylate to 2,3-dihydroxybutanedioate. The 2,3-dihydroxybutanedioate product has three enantiomers (L(+)-configuration, D(−)-configuration, and *meso*-configuration), formation of which is determined by the properties of the ORCH.

Many species have been reported to produce L(+)-dihydroxybutanedioate, including *Pseudomonas*, *Rhizobium*, *Corynebacterium*, *Acetobacter*, *Labrys*, *Rhodococcus*, *Nocardia*, and *Klebsiella* (Liu et al.,

‡ Corresponding author
* Project supported by the Zhejiang Provincial Natural Science Foundation of China (No. LQ19C200001), the Education Department of Zhejiang Province Scientific Research Project (No. Y201737925), the Zhejiang Provincial Key Laboratory for Chemical and Biological Processing Technology of Farm Products (No. 2016KF0048), and the Key Laboratory of Bioorganic Synthesis of Zhejiang Province (No. 20170110), China
# Electronic supplementary materials: The online version of this article (https://doi.org/10.1631/jzus.B1900392) contains supplementary materials, which are available to authorized users
ORCID: Zi-sheng LUO, https://orcid.org/0000-0001-8232-9739

2007a; Cheng et al., 2014a; Bao et al., 2015). The ORCHs from the latter three species have been characterized and their genes have been cloned (Liu et al., 2007b; Wang et al., 2013; Cheng et al., 2014b). They proceed through a two-step mechanism using a nucleophile-histidine-acid catalytic triad, involving the formation of a covalent intermediate between enzyme and substrate (Pan et al., 2011; Wang et al., 2013; Cheng et al., 2014b). A few genera have been reported to produce D(−)-dihydroxybutanedioate, including *Pseudomonas*, *Alcaligenes*, and *Bordetella* (Pan et al., 2008). The amino acid sequences of the ORCHs from the latter two have been determined (Pan et al., 2010). The crystal structure of ORCH from *Bordetella* and its possible catalytic mechanism have been reported (Bao et al., 2014; Dong et al., 2018). The only genus known to produce *meso*-dihydroxybutanedioate is *Flavobacterium* (Martin and Foster, 1955), but there have been no further reports on the gene sequence, enzyme properties, or catalytic mechanism of the enzyme(s) involved.

L(+)-Dihydrobutanedioic acid is the most abundantly produced dextrorotatory isomer of 2,3-dihydrobutanedioic acid. It is widely used in the food, textile, chemical, pharmaceutical, and building industries. Biocatalytic preparation by ORCH is a trend in L(+)-dihydrobutanedioic acid production. However, poor stability of reported ORCHs restricts the efficiency of L(+)-dihydrobutanedioic acid production. *Labrys* sp. WH-1 has been isolated by our laboratory, which produces L(+)-dihydroxybutanedioate and shows good production stability with high enantioselectivity. In this study, the ORCH of *Labrys* sp. WH-1 was purified, characterized, cloned, and expressed in *Escherichia coli*.

2 Materials and methods

2.1 Purification of ORCH from *Labrys* sp. WH-1

Labrys sp. WH-1 was stored by our laboratory and it was incubated aerobically at 30 °C for 24 h in a fermentation medium (1 g/L $K_2HPO_4{\cdot}3H_2O$, 5 mL/L corn steep liquor, 5 g/L saccharose, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 2.7 g/L NH_4Cl, and 10 g/L L(+)-tartrate, pH 8.0). Purification of ORCH from *Labrys* sp. WH-1 was performed by ammonium sulfate precipitation, diethylaminoethyl (DEAE)-Sepharose chromatography, phenyl-Sepharose chromatography, and Sephadex G100 chromatography at 4 °C, according to the method of Cheng et al. (2014a), except that the $(NH_4)_2SO_4$ precipitation ranged from 40% to 60% saturation. The content of dihydrobutanedioic was determined by high-performance liquid chromatography (HPLC) and the ORCH activity was assayed according to the method of Cheng et al. (2014a). The yield of each step was calculated, and the purified samples were analyzed by 12% (0.12 g/mL) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2 Molecular weight analysis

Purified ORCH was subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Sangon Biotech Co., Ltd., Shanghai, China) to determine the accurate molecular weight (Zhu et al., 2018). Purified ORCH was loaded on a Biosep SEC-s3000 column (300.0 mm× 7.8 mm) using an ÄKTA purification system (GE Healthcare, Uppsala, Sweden) at a flow rate of 1 mL/min and 4 °C to calculate the molecular mass of the nature protein in solution by size exclusion chromatography. Thyroglobulin (669.0 kDa), immunoglobulin A (380.0 kDa), immunoglobulin G (150.0 kDa), transferrin (80.0 kDa), ovalbumin (45.0 kDa), β-lactoglobulin (35.0 kDa), chymotrypsinogen (25.0 kDa), and ribonuclease A (13.7 kDa) were used as molecular mass standards.

2.3 Determination of isoelectric point

Capillary isoelectric focusing (CIEF) was used for determination of the isoelectric point (p*I*) of ORCH loaded on a Beckman Coulter P/ACE MDQ capillary electrophoresis system (Fullerton, CA, USA) according to the method by Salmanowicz et al. (2014). The apparent p*I* was calculated via linear regression analysis of marker p*I* values (p*I* values of 4.1, 5.5, 7.0, and 9.5; Beckman Coulter) versus migration time.

2.4 Characterization of ORCH

The effects of temperature, pH, metal ions, and other reagents on ORCH activity were determined. Kinetic parameters (K_m and k_{cat}) were determined at substrate concentrations from 10 to 200 mmol/L (Yuan et al., 2018). Circular dichroism (CD) spectra were measured on a Jasco-815 spectropolarimeter (Jasco, Japan), and the content of secondary structural

elements was predicted using the program K2D according to the method of Bao et al. (2014).

2.5 Protein terminal sequencing

The purified ORCH was electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The transferred protein band was subjected to N-terminal and C-terminal sequence analysis on an ABI Procise N sequencer (Applied Biosystems, CA, USA).

2.6 Cloning and expression of ORCH in *E. coli*

Genomic DNA of *Labrys* sp. WH-1 was extracted using an Ezup spin column bacterial genomic DNA isolation kit (Sangon Biothech, Shanghai, China). *ORCH* was amplified with degenerate primers P1 and P2 (Table S1), which were designed based on the sequences of the N-terminus and C-terminus of *ORCH*. The PCR products were purified and ligated with pUCm-T by using the T/A cloning procedure, and the recombinant plasmid was transformed into *E. coli* DH5α cells. A positive clone was sequenced. Primers P3 and P4 (Table S1) were designed to amplify *ORCH* and cloned into vector pTrc99A with *Nco*I and *Bam*HI. *ORCH* was overexpressed in *E. coli* JM109 cells with addition of isopropyl thiogalactoside (IPTG) at 30 °C for 18 h.

2.7 Nucleotide sequence accession number

The nucleotide sequence of *ORCH* from *Labrys* sp. WH-1 was submitted to the GenBank nucleotide sequence database (https://www.ncbi.nlm.nih.gov/genbank) under accession No. MK994516.

3 Results

3.1 Purification and analysis of ORCH

The ORCH from *Labrys* sp. WH-1 was purified with 12.7% yield by $(NH_4)_2SO_4$ precipitation and three chromatographic steps (Table 1). The specific activity increased from 8.9 to 231.7 U/mg, indicating a 26-fold purification of the enzyme. SDS-PAGE (Fig. 1a) revealed that the ORCH was essentially pure with only very faint bands of contaminating protein being visible, and the molecular mass of the ORCH was between 25 and 35 kDa. MALDI-TOF-MS analysis (Fig. 1b) showed that the accurate molecular weight was 30.2 kDa.

In size-exclusion chromatography (Fig. 1c), the purified ORCH from *Labrys* sp. WH-1 had one main peak with an elution time of 10.05 min, corresponding to an apparent molecular mass of 30 kDa. Given that the ORCH monomeric unit has a molecular mass of about 30 kDa (Figs. 1a and 1b); this suggests that ORCH from *Labrys* sp. WH-1 is a monomer in solution.

CIEF is a special mode of capillary electrophoresis that is used for the separation and charge-based characterization of protein and for the determination of p*I*. The mobilization stage from 15–38 min shows data for p*I* peptide markers and ORCH (Fig. 1d). The apparent p*I* of ORCH was calculated from a curve of the linear relationship between the detection time and the theoretical p*I* values of the peptide markers. The experimental p*I* value of the ORCH was determined to be 8.57 with retention time of 19.29 min.

3.2 Characterization of ORCH

As Fig. 2a shows, the ORCH from *Labrys* sp. WH-1 was most active between 50 and 60 °C with an optimum temperature for activity of 55 °C, and the enzyme was stable at up to 55 °C for 30 min. The temperature at which the activity of the enzyme decreased by 50% in 15 min (T_{50}^{15}) and the half-life at 50 °C ($t_{1/2,\ 50\ °C}$) of ORCH were about 61 °C (Fig. 2b) and 51 min (Fig. 2c), respectively. As shown in Fig. 2d, the ORCH showed high activity between pH 7.5 and 9.5 with optimal pH for activity of 8.5. The ORCH showed high stability in a broad pH range, from 4.0 to 10.0 (Fig. 2d).

As Fig. 2e shows, the ORCH from *Labrys* sp. WH-1 retained high relative activity in the presence

Table 1 Summary of purification of ORCH from *Labrys* sp. WH-1

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme	15903	1796.0	8.9	100.0	1.0
$(NH_4)_2SO_4$	10982	896.0	12.3	69.1	1.4
Diethylaminoethyl (DEAE)-Sepharose	7681	188.0	40.9	48.3	4.6
Phenyl-Sepharose	5662	28.9	195.9	35.6	22.0
Sephadex G100	2016	8.7	231.7	12.7	26.0

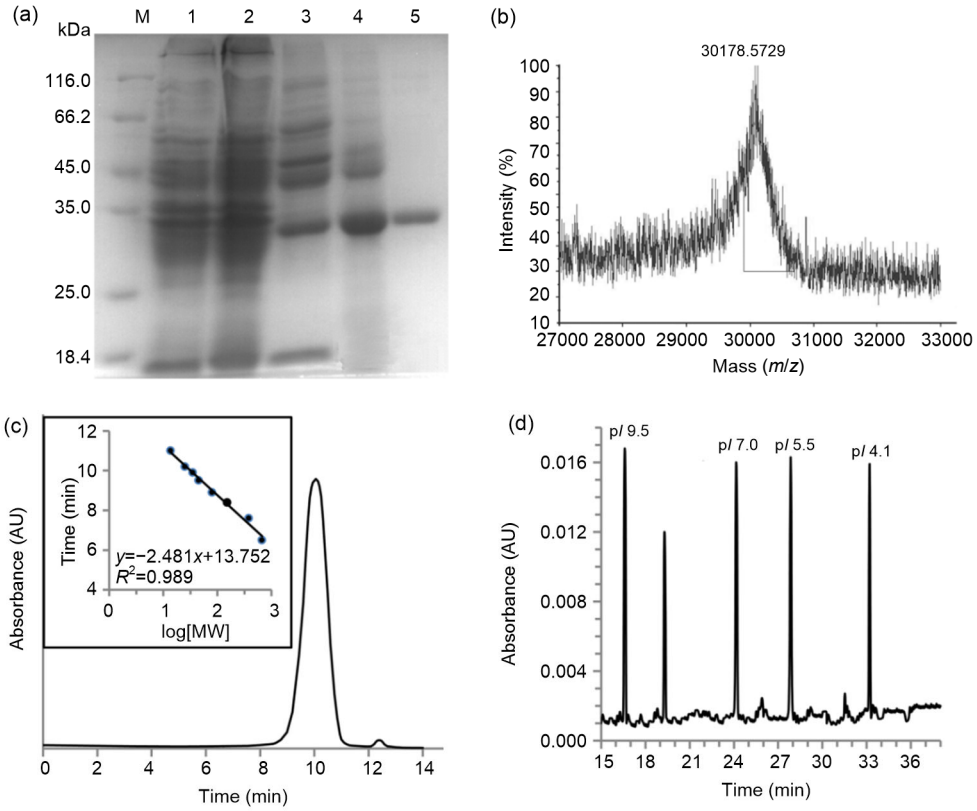

Fig. 1 Purification and analysis of ORCH from *Labrys* sp. WH-1

(a) Purification of the oxiranedicarboxylate hydrolase (ORCH) from *Labrys* sp. WH-1. Samples were taken at different stages of ORCH purification (Table 1) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lanes: M, protein molecular weight standards; 1, crude enzyme; 2, 40%–60% saturated $(NH_4)_2SO_4$ precipitate; 3, pooled fractions after diethylaminoethyl (DEAE)-Sepharose chromatography; 4, pooled fractions after phenyl-Sepharose chromatography; 5, pooled fractions after Sephadex G100 chromatography. (b) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of the ORCH from *Labrys* sp. WH-1. (c) Size exclusion chromatography profile. The peak at 10.05 min corresponds to a molecular mass of 30 kDa. Inset, the standard curve (see Section 2.1). (d) Capillary isoelectric focusing profile of ORCH from *Labrys* sp. WH-1 and standard p*I* markers

of a metal chelating agent (20 mmol/L ethylenediaminetetraacetic acid (EDTA)-Na_2), indicating that it is a metal-independent enzyme. It was strongly inhibited by Cu^{2+} and Ag^{+}. The ORCH was not stable in the presence of anionic surfactants (SDS and sodium deoxycholate), while it was only slightly affected by zwitterionic surfactants (Triton X-100 and Tween 80), suggesting that zwitterionic surfactants may be more suitable for increasing cell permeability during catalytic conversion by free cells. Dithiothreitol (DTT) is a powerful reducing agent, which can protect the reducing groups (such as –SH) of enzymes and block the formation of disulfide bonds. DTT only slightly affected the activity of the ORCH, suggesting that the enzyme may not contain disulfide bonds. Other metal ions (Fe^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, Al^{3+}, Mn^{2+}, Co^{2+}, Ba^{2+}, and Zn^{2+}) and ethanol could weakly affect the activity of the enzyme.

Kinetic parameters of the ORCH for disodium *cis*-2,3-oxiranedicarboxylate were determined in the optimal reaction conditions (55 °C, pH 8.5). The values of K_m, k_{cat}, and k_{cat}/K_m were calculated to be 18.7 mmol/L, 222.3 s^{-1}, and 11.9 mmol/(L·s), respectively, which showed stronger affinity toward *cis*-2,3-oxiranedicarboxylate and higher catalytic efficiency.

Ultraviolet (UV) CD may be used to investigate the secondary structure of proteins. CD spectra of the ORCH are shown in Fig. S1, and the secondary structural content of the protein was predicted using the software K2D. The predicted contents of helix and sheet in ORCH were 40.3% and 12.5%, respectively.

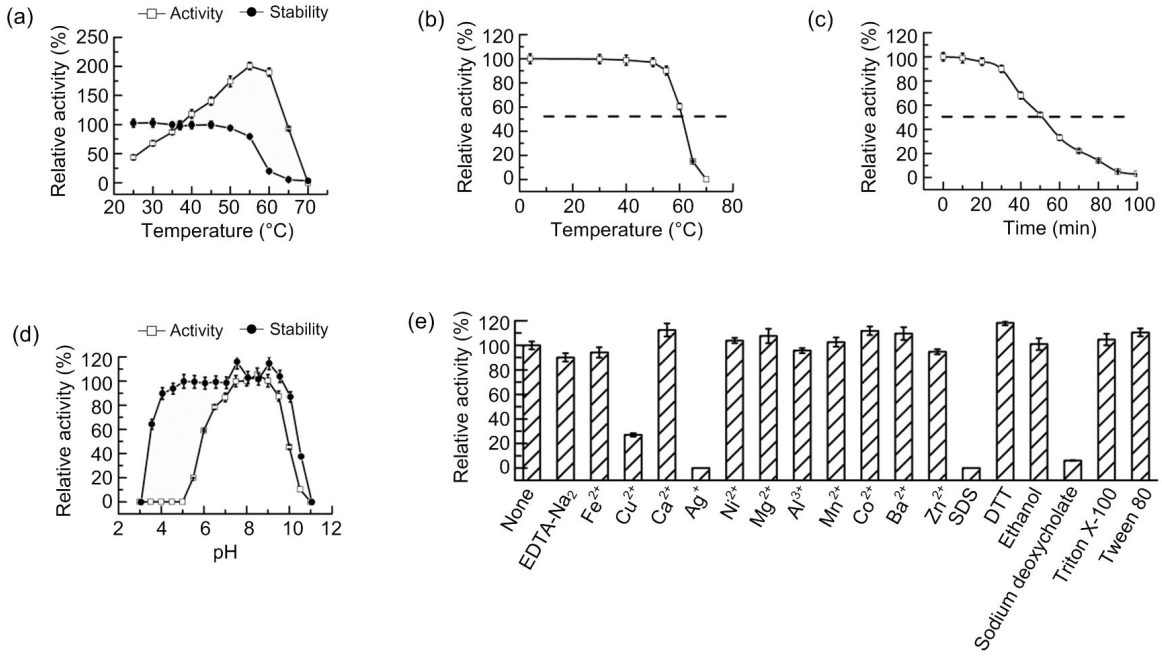

Fig. 2 Effects of temperature, pH, and various reagents on the activity of ORCH from *Labrys* sp. WH-1

(a) The optimum temperature for enzyme activity and the thermal stability of purified oxiranedicarboxylate hydrolase (ORCH). The optimum temperature was determined by varying the temperature at pH 8.0. Thermal stability was determined after pre-incubation of purified ORCH at different temperatures for 30 min at pH 8.0. (b) The temperature at which the activity of the enzyme decreased by 50% in 15 min (T_{50}^{15}) was determined as follows: purified ORCH was heated for 15 min at different temperatures in thin-walled tubes, cooled immediately on ice, and the residual activity was measured. (c) The half-life at 50 °C ($t_{1/2,\ 50\ °C}$) was determined by incubating purified ORCH in a water bath at 50 °C for various time, cooling immediately in an ice-water bath, and measuring the residual activity. (d) The optimum pH for enzyme activity and the pH stability of purified ORCH. The optimum pH was determined by assay at various pH values at 37 °C. pH stability was investigated by preincubation of ORCH in different buffers for 30 min at 25 °C. (e) Effects of metal ions (1 mmol/L) and chemicals (0.1% (1 g/L), except for 20 mmol/L ethylenediaminetetraacetic acid (EDTA)-Na_2) on the activity of purified ORCH. The activity of the ORCH from *Labrys* sp. WH-1 in standard reaction conditions (37 °C, pH 8.0) was (231±10.3) U/mg, and the residual activity was taken as 100%. Each value is expressed as mean±standard deviation (SD), n=3. SDS: sodium dodecyl sulfate-polyacrylamide; DTT: dithiothreitol

3.3 Protein terminal sequencing

Protein terminal sequence analysis was determined at the N-terminal and C-terminal ends of the ORCH, separately. The N-terminal amino acid sequence was MKFSGASLFA, and the C-terminal was FTELADRLGA.

3.4 Cloning and expression of *ORCH*

Based on the result of the N-terminal and C-terminal sequence analysis, degenerate primers P1 and P2 (Table S1) were designed to amplify the *ORCH* gene, and a sequence of about 0.8 kb was achieved. The sequence analysis using DNAMAN Version 6.0 (LynnonBiosoft, CA, USA) indicated that the fragment contained an open reading frame (ORF) of 825 bp encoding 274 amino acid residues (GenBank accession No. MK994516). The deduced molecular mass and p*I* of ORCH from *Labrys* sp. WH-1 were 30.2 kDa and 8.43, respectively, which were consistent with the actual measured results (Figs. 1c and 1d). *ORCH* was over expressed in *E. coli* JM109 and the recombinant bacteria showed the high-level expression of recombinant protein induced by IPTG (Fig. S2). The enzyme activity of engineering bacteria could achieve (128200±7400) U/g biomass, which was about 33 times as much as that of the original strain ((3800±360) U/g biomass). This established a beneficial foundation for industrial production of L(+)-dihydrobutanedioic acid.

4 Discussion

The ORCH from *Labrys* sp. WH-1 was purified and some characteristics were determined in the study. The ORCHs from *Rhodococcus* sp. ML-0004 (Liu et al., 2007a) and *Klebsiella* sp. BK-58 (Cheng et al., 2014a) were reported to produce L(+)-dihydrobutanedioic acid, and are commonly used strains in industrial production. The properties of ORCHs from these different sources (Liu et al., 2007a; Pan et al., 2011; Cheng et al., 2014a, 2014b; Qiao et al., 2015) are compared and summarized in Table 2. The ORCH from *Labrys* sp. WH-1 is a monomer in solution and is a metal-independent enzyme, similar to the other two ORCHs. The p*I* of ORCH from *Labrys* sp. WH-1 is higher than those from *Rhodococcus* and *Klebsiella*, which were predicted from their amino acid sequences using Compute pI/Mw software (https://web.expasy.org/compute_pi).

ORCH from *Labrys* sp. WH-1 was more thermostable than the ORCHs from *Rhodococcus* and *Klebsiella* in terms of optimum temperature, T_{50}^{15}, and $t_{1/2,\ 50\ °C}$ (Qiao et al., 2015). The activity range of ORCH from *Labrys* sp. WH-1 was wider than that of the ORCH from *Rhodococcus* and similar to that of the ORCH from *Klebsiella*. The optimum pH for each of these L(+)-dihydroxybutanedioate-producing ORCHs was moderately alkaline. The mechanism of the ORCHs from *Rhodococcus* (Pan et al., 2011) and *Klebsiella* (Cheng et al., 2014b) was proposed to involve a two-step reaction involving the formation of a covalent intermediate between enzyme and substrate, which was easier to generate in weakly alkaline conditions. Although the ORCH from *Rhodococcus* had a higher k_{cat}, it had a lower affinity for substrate (K_m), resulting in a lower enzyme efficiency (k_{cat}/K_m) (Liu et al., 2007b). The k_{cat}/K_m of the ORCH from *Labrys* sp. WH-1 was the best among the reported L(+)-dihydroxybutanedioate-producing ORCHs.

Taken together, although the ORCH from *Rhodococcus* had the highest enzyme activity, its thermal and pH stability were the poorest. The properties of the ORCH from *Klebsiella* are intermediate between those of the enzymes from *Labrys* and *Rhodococcus*. The ORCH from *Labrys* sp. WH-1 showed the best catalytic efficiency and stability (thermal and pH stability), and is less sensitive to most metal ions and chemicals, indicating that it could be a good industrial biocatalyst for production of L(+)-dihydrobutanedioic acid. *ORCH* from *Labrys* sp. WH-1 was further cloned and its amino acid sequence was aligned with those from *Rhodococcus* and *Klebsiella* using ClustalW2 program (Fig. 3). It showed 37% and 47% identity with the ORCHs from *Rhodococcus* and *Klebsiella*, respectively. The amino acid composition of these three ORCHs is shown in Table S2. The ORCH from *Labrys* has higher basic amino acid content (Arg, Lys, and His) than the other two, consistent with its higher isoelectric point (Fig. 1d). The ORCH from *Labrys* sp. WH-1 has the highest hydrophobic amino acid content (Ala, Val, Ile, Leu, Phe, and Trp) and the lowest polar amino acid content (Cys, Ser, Thr, Tyr, Asn, and Gln) of these three enzymes, indicating that it has the best stability, which was confirmed by our experiments (the hydrophobic amino acid content is related to protein stability). The

Table 2 Comparison of properties of L(+)-dihydrobutanedioic acid-producing ORCHs from different species

Species	Molecular weight (kDa)	Subunit	Optimum temperature (°C)	Thermal stability (°C)	T_{50}^{15} (°C)	$t_{1/2,\ 50\ °C}$ (min)	Optimal pH	Range of pH stability
Rhodococcus	28.1	Monomer	37	<35	44	8.5	7.5	7–8
Klebsiella	30.1		50	<50			8.5	5–10
Labrys	30.2	Monomer	55	<55	61	51.0	8.5	4–10

Species	Metal-dependence	K_m (mmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m (mmol/(L·s))	p*I*	Conversion rate (%)	EE value (%)	Reference
Rhodococcus	No	43.3	290.1	6.7	4.75[a]	>99.0	>99.5	Liu et al., 2007a; Pan et al., 2011; Qiao et al., 2015
Klebsiella	No	19.6	217.7	11.1	7.78[a]	>99.0	>99.5	Cheng et al., 2014a, 2014b
Labrys	No	18.7	222.3	11.9	8.57	>99.0	>99.5	This study

T_{50}^{15}: temperature at which the activity of the enzyme decreased by 50% in 15 min; $t_{1/2,\ 50\ °C}$: half-life at 50 °C; K_m, k_{cat}: kinetic parameters; EE: enantiomeric excess. [a] Isoelectric point (p*I*) was predicted from the amino acid sequence using Compute pI/Mw software

Fig. 3 Amino acid sequence alignments for ORCHs from different species

The sequences of oxiranedicarboxylate hydrolases (ORCHs) from *Labrys* sp. WH-1 (this study), *Klebsiella* sp. BK-58 (GenBank accession No. KF977193) and *Rhodococcus* sp. ML-0004 (GenBank accession No. DQ471957) were aligned with ClustalW2. Identical amino acids are marked with an asterisk, conserved substitution residues with a colon, semiconserved substitution residues with a period. The predicted secondary structure elements of ORCHs by PredictProtein are shown. α-Helices are shaded black and β-sheets are shaded gray. The indispensable catalytic amino acids for *Klebsiella* and *Rhodococcus* are shown in the box

secondary structures of ORCHs were predicted by PredictProtein (http://www.predictprotein.org), and it gave similar results to those from the amino acid sequence alignment data (Fig. 3), except for N-terminal sequences. UV CD was used to investigate the secondary structure, and the contents of helix and sheet in ORCH from *Labrys* sp. WH-1 were 40.3% and 12.5%, respectively, which is consistent with software predictions. The nine residues for ORCH from *Labrys* sp. WH-1 (D48, T52, R85, N165, K195, Y201, A219, H221, and D224) are completely conserved with the important catalytic residues for ORCHs from *Rhodococcus* (D18, T22, R55, N134, K164, Y170, A188, H190, and D193) and *Klebsiella* (D48, T52, R85, N165, K195, Y201, A219, H221, and D224), suggesting that they may adopt the same catalytic mechanism (Pan et al., 2011; Cheng et al., 2014b).

5 Conclusions

The ORCH from *Labrys* sp. WH-1 was cloned and some characteristics were determined and compared with those of other reported ORCHs. Its catalytic efficiency and thermal stability were the best among the reported L(+)-dihydroxybutanedioate-producing ORCHs. Sequence alignments showed that the residues involved in catalysis were conserved. As these L(+)-dihydroxybutanedioate-producing ORCHs have the same or similar functions, we can in principle engineer a novel ORCH enzyme with high catalytic activity and good stability by comparison between ORCH sequences and molecular structure modification, combining the favorable characteristics of the ORCH from each source. Achieving this will require knowledge of their 3D structures, which may be the top priority in future research into ORCH enzymes.

Contributors

Wen-na BAO and Zi-sheng LUO designed the research and wrote the manuscript. Shi-wang LIU polished the English. Yuan-feng WU and Gong-nian XIAO analyzed the data. Pei-lian WEI and Yong LIU performed the experiments. All authors read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethical guidelines

Wen-na BAO, Zi-sheng LUO, Shi-wang LIU, Yuan-feng WU, Pei-lian WEI, Gong-nian XIAO, and Yong LIU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Bao WN, Pan HF, Zhang ZH, et al., 2014. Analysis of essential amino acid residues for catalytic activity of *cis*-epoxysuccinate hydrolase from *Bordetella* sp. BK-52. *Appl Microbiol Biotechnol*, 98(4):1641-1649. https://doi.org/10.1007/s00253-013-5019-2

Bao WN, Pan HF, Zhang ZH, et al., 2015. Isolation of the stable strain *Labrys* sp. BK-8 for L(+)-tartaric acid production. *J Biosci Bioeng*, 119(5):538-542. https://doi.org/10.1016/j.jbiosc.2014.10.013

Cheng YQ, Wang L, Pan HF, et al., 2014a. Purification and characterization of a novel *cis*-epoxysuccinate hydrolase from *Klebsiella* sp. that produces L(+)-tartaric acid. *Biotechnol Lett*, 36(11):2325-2330. https://doi.org/10.1007/s10529-014-1614-2

Cheng YQ, Pan HF, Bao WN, et al., 2014b. Cloning, homology modeling, and reaction mechanism analysis of a novel *cis*-epoxysuccinate hydrolase from *Klebsiella* sp. *Biotechnol Lett*, 36(12):2537-2544. https://doi.org/10.1007/s10529-014-1638-7

Dong S, Liu X, Cui G, et al., 2018. Structural insight into the catalytic mechanism of a *cis*-epoxysuccinate hydrolase producing enantiomerically pure D(−)-tartaric acid. *Chem Commun (Camb)*, 54(61):8482-8485. https://doi.org/10.1039/c8cc04398a

Liu ZQ, Li Y, Ping LF, et al., 2007a. Isolation and identification of a novel *Rhodococcus* sp. ML-0004 producing epoxide hydrolase and optimization of enzyme production. *Process Biochem*, 42(5):889-894. https://doi.org/10.1016/j.procbio.2007.01.009

Liu ZQ, Li Y, Xu YY, et al., 2007b. Cloning, sequencing, and expression of a novel epoxide hydrolase gene from *Rhodococcus opacus* in *Escherichia coli* and characterization of enzyme. *Appl Microbiol Biotechnol*, 74(1):99-106. https://doi.org/10.1007/s00253-006-0635-8

Martin WR, Foster JW, 1955. Production of *trans*-L-epoxysuccinic acid by fungi and its microbiological conversion to *meso*-tartartic acid. *J Bacteriol*, 70(4):405-414.

Pan HF, Xie ZP, Bao WN, et al., 2008. Isolation and identification of a novel *cis*-epoxysuccinate hydrolase-producing *Bordetella* sp. BK-52 and optimization of enzyme production. *Acta Microbiol Sin*, 48(8):1075-1081 (in Chinese). https://doi.org/10.13343/j.cnki.wsxb.2008.08.012

Pan HF, Bao WN, Xie ZP, et al., 2010. Molecular cloning and characterization of a *cis*-epoxysuccinate hydrolase from *Bordetella* sp. BK-52. *J Microbiol Biotechnol*, 20(4):659-665. https://doi.org/10.4014/jmb.0905.05059

Pan HF, Xie ZP, Bao WN, et al., 2011. Site-directed mutagenesis of epoxide hydrolase to probe catalytic amino acid residues and reaction mechanism. *FEBS Lett*, 585(15):2545-2550. https://doi.org/10.1016/j.febslet.2011.07.006

Qiao P, Wu MB, Zhu L, et al., 2015. Enhancing the thermal tolerance of a *cis*-epoxysuccinate hydrolase via combining directed evolution with various semi-rational redesign methods. *J Mol Catal B Enzym*, 121:96-103. https://doi.org/10.1016/j.molcatb.2015.08.010

Salmanowicz BP, Langner M, Franaszek S, 2014. Charge-based characterisation of high-molecular-weight glutenin subunits from common wheat by capillary isoelectric focusing. *Talanta*, 129:9-14. https://doi.org/10.1016/j.talanta.2014.04.055

Wang ZQ, Wang YS, Su ZG, 2013. Purification and characterization of a *cis*-epoxysuccinic acid hydrolase from *Nocardia tartaricans* CAS-52, and expression in *Escherichia coli*. *Appl Microbiol Biotechnol*, 97(6):2433-2441. https://doi.org/10.1007/s00253-012-4102-4

Yuan L, Sadiq FA, Liu TJ, et al., 2018. Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 19(8):630-642. https://doi.org/10.1631/jzus.B1700352

Zhu J, Zhu LWS, Yang JH, et al., 2018. Proteomic analysis of human umbilical vein endothelial cells exposed to $PM_{2.5}$. *J Zhejiang Univ-Sci B (Biomed & Biotechnol)*, 19(6):458-470. https://doi.org/10.1631/jzus.B1700103

List of electronic supplementary materials

Table S1 Primers used in this study

Table S2 Amino acid composition of ORCHs from *Labrys* sp. WH-1 and other reported species

Fig. S1 CD spectra of the ORCH from *Labrys* sp. WH-1

Fig. S2 Expression of ORCH from *Labrys* sp. WH-1 in recombinant bacteria by SDS-PAGE analysis

中文概要

题　目：双头菌 WH-1 环氧乙烷二酸水解酶的克隆和酶学性质的研究

目　的：克隆双头菌 WH-1 环氧乙烷二酸水解酶（ORCH）的基因并研究其酶学性质。

创新点：首次获得双头菌 ORCH 的基因，且该酶催化效率高，热稳定性好。

方　法：柱层析纯化 ORCH 后，进行酶学性质研究；通过蛋白末端测序和 PCR 获得其基因序列；通过二级结构预测和多序列比对进行 ORCH 序列分析。

结　论：来源于双头菌 WH-1 的 ORCH 是迄今报道的催化效率和热稳定性最好的 ORCH，为 L(+)-2,3-二羟基丁二酸的生产提供了新的催化剂。

关键词：环氧乙烷二酸水解酶；L(+)-2,3-二羟基丁二酸；酶学性质；克隆