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Highly efficient resolution of mandelic acid using lipase from *Pseudomonas stutzeri* LC2-8 and a molecular modeling approach to rationalize its enantioselectivity

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ABSTRACT

Mandelic acid, a key precursor of chiral synthons, was successfully acylated in diisopropyl ether. The reaction was catalyzed by the lipase from *Pseudomonas stutzeri* LC2-8, and vinyl acetate was employed as acyl donor. Under the optimized reaction conditions, a resolution of 180 mM (55 g/L) mandelic acid was achieved. (*S*)-*O*-Acetyl mandelic acid was enantioselectivity formed in >99% *ee* at a yield close to the maximum theoretical value for kinetic resolution (50%). The highly substrate tolerable and enantioselective nature of lipase LC2-8 suggests that it is of great potential for the practical resolution of racemic mandelic acid. Additionally, the high enantiopreference of lipase LC2-8 toward (*S*)-mandelic acid in acetylation was also rationalized through molecular docking and molecular dynamics simulations.

1. Introduction

Enantiomers of mandelic acid and their derivatives have been considered as important substances because they are utilized extensively for synthetic purposes as well as in stereochemical investigations. Mandelic acid enantiomers are employed in the resolution of racemic alcohols and amines [1,2]. In addition, (*R*)-mandelic acid is used as a versatile intermediate for preparation of semisynthetic cephalosporins, penicillins, anti-tumor agents and anti-obesity agents [3]. In the last decade, the use of lipases in the enantioselective resolution of racemic mandelic acid for obtaining chiral enantiomers has received considerable attention. Although a high enantiomeric excess (*ee* value) can be obtained in some reports [4–7], the relatively low substrate concentration, usually below 50 mM, restricts its practical application. Therefore, developing the efficient resolution process and improving its space–time yield become imperative.

With the rapid development of computer simulations at present, biomolecular modeling provides a promising way to understand the mechanism and selectivity of enzyme-catalyzed reactions and to guide the modification of enzymes. For example, using docking simulations, Santaniello succeeded in explaining the enantioselectivity of the *Burkholderia cepacia* lipase-catalyzed transesterification of aromatic primary alcohols with vinyl esters with different chain lengths [8]. Based on molecular dynamics simulation, using methyl mandelate as a "model" substrate, Yu's group has successfully shed light on the source of enantioselectivity modified and the trade-off of enantioselectivity and activity in directed evolution of an esterase from *Rhodobacter sphaerioides* [9,10]. Thus, molecular modeling seems to be a good choice to rationalize the enantioselectivity of the lipase-catalyzed acylation of mandelic acid.

Recently, we screened and characterized lipase LC2-8 from *Pseudomonas stutzeri* [11]. This lipase exhibited significant solvent tolerance and good enantioselectivity toward 1-phenylethanol. In the present work, lipase LC2-8 was used in the resolution of mandelic acid, with vinyl acetate as acyl donor (Fig. 1). Lipase LC2-8 showed high substrate concentration tolerance and excellent enantiopreference toward (*S*)-mandelic acid. In addition, we developed a molecular model and explained the strict enantioselectivity of lipase LC2-8 toward mandelic acid.

1.1. Biological and chemical materials

Lipase LC2-8 from *P. stutzeri* LC2-8 was screened and characterized in our previous report [11]. The strain of *P. stutzeri* LC2-8

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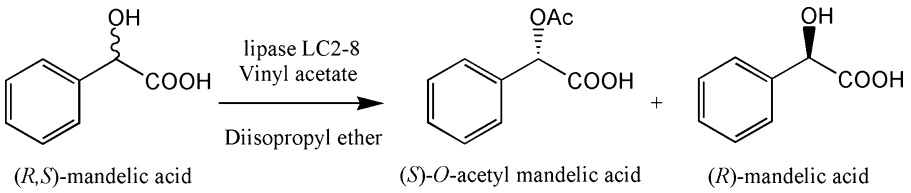

Fig. 1. Resolution of (*R,S*)-mandelic acid catalyzed by lipase LC2-8.

is currently deposited at the China Center for Type Culture Collection (Wuhan, China) with accession number CCTCC M 2010279. The nucleotide sequence of lipase LC2-8 has been assigned a GenBank accession number of JN681265.

Mandelic acid was purchased from Merck (Germany). *p*-Nitrophenyl palmitate (p-NPP) and high-performance liquid chromatography (HPLC)-grade solvents were purchased from Sigma (USA). All other chemicals were of analytical grade.

1.2. Preparation of crude lipase LC2-8

Strain LC2-8 was cultured in lipase-producing medium consisting of (w/v) 1.0% yeast extract, 0.8% glucose, 0.2% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% (v/v) Triton X-100, and 0.5% (v/v) sunflower oil, at a pH of 8.0. The incubations were carried out with shaking at 180 rpm at 30 °C. After 48 h of growth, the culture supernatant was collected by centrifugation. Chilled acetone was added under magnetic stirring to the crude lipase at 0 °C for 4 h until the ratio was 0.8:1 (v/v). The precipitate was obtained by centrifugation at 10,000 × *g* and 4 °C for 30 min. Finally, the precipitate was air-dried and lipase LC2-8 powder was obtained. The lipase activity was determined using a modified spectrophotometric method with p-NPP as substrate [12].

1.3. Resolution of mandelic acid catalyzed by lipase LC2-8

The resolution of (*R,S*)-mandelic acid was carried out in isopropyl ether containing 300 mM vinyl acetate and 30 mM mandelic acid. The reaction was carried out with shaking at 180 rpm, and was catalyzed by 10 mg/mL (30 U/mL) lipase LC2-8 powder at 30 °C. A control reaction was done by performing the above procedure in the absence of enzyme.

1.4. Enantiomeric analysis of products by HPLC

The concentrations of (*R,S*)-mandelic acid, (*S*)-, and (*R*)-*O*-acetyl mandelic acid were determined by HPLC (Dionex P680) using a Chiracel OD-H column (250 mm × 4.6 mm) and *n*-hexane/isopropanol/TFA (94/6/0.2, v/v/v) as mobile phase, which was introduced at a flow rate of 1 mL/min. The wavelength of the UV detector was set at 228 nm. The enantiomeric excess (ee_p, ee_s) and conversion yield (c) were calculated using the corresponding peak areas:

Enantiomeric excess,

$$ee_p = \frac{[(S)\text{-}O\text{-acetyl mandelic acid}] - [(R)\text{-}O\text{-acetyl mandelic acid}]}{[(S)\text{-}O\text{-acetyl mandelic acid}] + [(R)\text{-}O\text{-acetyl mandelic acid}]}$$

$$ee_s = \frac{[(R)\text{-mandelic acid}] - [(S)\text{-mandelic acid}]}{[(S)\text{-mandelic acid}] + [(R)\text{-mandelic acid}]}$$

Conversion yield [13],

$$c = \frac{ee_s}{ee_s + ee_p}$$

All reported data are averages of experiments performed at least in triplicate.

1.5. Construction of the homology model of lipase LC2-8

Lipase LC2-8 is composed of 311 amino acid residues including a predicted 24-amino acid signal peptide. All computational methods were carried out using DS2.5 (Accelrys Software Inc., San Diego, CA) [14]. A search of the NCBI database was done using the Basic Local Alignment Search Tool (BLAST) [15]. Automated sequence alignment between lipase LC2-8 and the template sequence was carried out. The BLAST search showed significant sequence identity (78.9%) between lipase LC2-8 and *Pseudomonas aeruginosa* lipase (Protein Data Bank (PDB) code: 1EX9) possessing the highest bit score of 450.7. Unanimously, the crystal structure of PAL (resolution 2.5 Å) was selected as the template. The generated structure was improved by subsequent refinement of the loop conformations by assessing the compatibility of an amino acid sequence to known PDB structures using the Protein Health module [14]. The geometry of loop regions was corrected using the Refine Loop. The three-dimensional structure of lipase LC2-8 obtained was further optimized by energy minimization. The steepest-descent method was set at 500 steps for first minimization, and was followed by 1000 steps of the conjugate gradient method. The programs PROCHECK [16] and Profiles-3D [17] were used to validate the accuracy of the refined model of lipase LC2-8.

1.6. Docking and molecular dynamics simulations

It is known that the transesterification mechanism is based on the acylation and deacylation of the catalytic triad of serine, and involves two tetrahedral intermediates. The first intermediate results from the nucleophilic attack of the catalytic serine on the acylating substrate, and forms the acyl–enzyme complex. The second derives from the nucleophilic attack of the acyl acceptor substrate on the acyl–enzyme complex, and leads to the release of the ester product [18]. Therefore, the Ser82 residue is covalently bound to the acyl group in order to mimic the acyl–enzyme complex [8,19,20].

The acylation of Ser82 was constructed using the Build fragment module of DS2.5. All hydrogen atoms were added in their theoretical positions. The catalytic residues Asp228 and His250 were assigned as deprotonated. After all preparations, the acyl–enzyme complex was run through 1000 steps of conjugate gradient energy minimization with the backbone atoms of the enzyme fixed, using the CHARMM force field.

The docking experiment was performed using the protocol of Dock Ligand (LibDock) (Receptor–Ligand Interactions module in DS2.5) [21]. The acyl–enzyme complex was used as receptor and the active site side chain residues were defined as the binding site sphere. The ligands with optimized structures were docked into the active site of the enzyme. The generated conformations were manually analyzed, and the one corresponding to the lowest energy and the highest consensus score was selected for the subsequent energy minimizations.

Considering that the docking algorithm treats the protein as a rigid body, a careful post-docking optimization was carried out on the enzyme–substrate complexes, in order to take into account a potential induced fit effect (little displacements in the protein