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A new mechanism of enantioselectivity toward chiral primary alcohol by lipase from *Pseudomonas cepacia*

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ABSTRACT

The stereo-recognition of chiral primary alcohols by lipase from *Pseudomonas cepacia* was found to deviate from earlier observations. Enantioselectivity toward 14 pairs of chiral primary alcohol esters by this lipase was dependent on the existence of an $O^{non\text{-}\alpha}$ (oxygen at non-α-position of the acyloxy group) in the alcohol moiety, and decreased as the size of the acyl moiety increased. Chemical modification on the lipase and molecular dynamics simulations indicated that Tyr^{29} located within the catalytic cavity forms a hydrogen bond with the $O^{non\text{-}\alpha}$ of the preferred enantiomer of the primary alcohol ester. However, a larger acyl moiety suffered stronger hindrance from the catalytic cavity wall of the lipase, pushing the $O^{non\text{-}\alpha}$ away from Tyr^{29}, and thus weakening the stereo-recognition.

1. Introduction

Chiral alcohols, including secondary and primary alcohols, are a common type of optically active chemicals. As chiral building blocks or chiral sources, secondary and primary alcohols play important roles in medicine, cosmetic production, and food chemistry. Lipase-catalyzed resolution is an effective method to produce optically pure enantiomers [1–3]. Therefore, the mechanism of chiral-recognition of alcohols by lipases has become a research hotspot over the last few years. Kazlauskas et al. [4] investigated the resolution of 94-pairs of enantiomers of chiral alcohols catalyzed by lipases including *Pc*L (*Pseudomonas cepacia* lipase), and proposed an empirical law of enantiopreference: the stereo-specific catalytic cavity of the enzyme contains large and small pockets that fit, respectively, the large and small group on the chiral center of the preferred enantiomer. This groundbreaking law was considered to be the origin of enantioselectivity by lipases [5,3]. Based on this law and using various genetic approaches, a number of studies on improving, manually altering lipase's enantioselectivity and even reversing the enantiopreference were reported [6–8]. Moreover, researchers were able to quantitatively predict with reasonable precision the enantioselectivity ratio of particular lipase-catalyzed resolution of chiral compounds using modern computational tools such as molecular docking and QSAR (quantitative structure–activity relationship) calculation [9,10].

Nonetheless, the success of these studies was only based on secondary alcohols, whose chiral center is the hydroxyl α-C of the alcohol, the C^{α}_{alc}. Research, either genetic or computational, on stereo-recognition of primary alcohols, whose chiral center is not the C^{α}_{alc}, has been limited. These alcohols could only be resolved by a limited number of lipases including *Pc*L [11,12]. Although by these lipases, only a small number of primary alcohols could be recognized, and even toward this small number of alcohols, the lipases' enantiopreference did not obey Kazlauskas' Rule [13–17]. In the present contribution, enantioselectivity by *Pc*L toward 14 pairs of different chiral primary alcohol esters was investigated. The mechanism behind the deviant behavior of stereo-recognition of these substrates was studied by chemical modification of *Pc*L, MALDI-TOF mass spectrometry, and molecular dynamics simulation. As a result, a new mechanism for enantioselectivity of lipase is proposed.

2. Materials and methods

2.1. Materials

*Pc*L (*Pseudomonas cepacia* lipase in powder form, commercially Lipase PS) was purchased from Amano Enzyme Inc. Chiral primary alcohols including 2, 3, 3-trimethyl-1-butanol, 2-phenyl-1-propanol, 2-phenyl-1-butanol, 2-methoxymethyl-1-butanol, 2-methoxy-1-butanol and 2-phenoxy-1-propanol together with

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analytical samples of their optically pure enantiomers were purchased from Tokyo Chemical Industry Co. Ltd. Modification reagents, including CHD (1, 2-cyclohexanedione); DEPC (diethyl pyrocarbonate); EDA (ethylenediamine); EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride); HNBB (2-hydroxy-5-nitrobenzyl bromide); IAA (iodoacetic acid); ICl (iodine monochloride); NAI (*N*-acetylimidazole); TNBS (2, 4, 6-trinitrobenzenesulfonic acid); TNM (tetranitromethane), were also purchased from Tokyo Chemical Industry Co. Ltd. All other chemicals referred in this paper were of analytical grade.

2.2. Synthesis of substrates

Esters of chiral primary alcohols were synthesized from their corresponding alcohols. Alcohols and triethylamine (molar ratio = 1:1.2) were mixed in an ice bath, and acetyl chloride was slowly added. The reaction mixture was stirred overnight. The end of reaction was determined by TLC (thin layer chromatography). The product was washed with a saturated sodium bicarbonate solution several times, and residual water was removed by anhydrous sodium sulphite.

2.3. Resolution of primary alcohol esters

10 mL of a mixed solution of crude *Pc*L powder (10 mg mL^{-1}), substrate (ester of chiral primary alcohol, 100 mmol L^{-1}) and PBS (phosphate buffer solution, 100 mmol L^{-1}, pH 7.0, including 10% *V*/*V* isopropanol as co-solvent) was added into a 20 mL flask incubated at 27 °C (300 K) under 200 rpm stirring. The reaction time was controlled as the conversion ratio was between 20 and 30%. The product was extracted by an equal volume of *n*-octanol. Substrates with aryl groups (**2**–**7**, **10**–**14**) were analyzed by an Agilent 1100 HPLC equipped with an Chiralpak AD-H column, whereas those without an aryl group (**1**, **8** and **9**) were analyzed by an HP 1890 GC equipped with a Varian CP-Cyclodextrin-B-2, 3, 6-M-19 capillary GC column. The initial reaction rate = $c{\cdot}[\text{Ester}]_0{\cdot}V/\Delta t$, where c is the conversion of the substrate, herein c should be less than 5%; $[\text{Ester}]_0$ is the initial molar concentration of the substrate (100 mmol L^{-1}); V is the volume of the reactant (10 mL); and Δt is the reaction time. The enantioselectivity ratio (E value) was calculated according to Chen et al. [18]: $E = \ln\{1 - c(1 + ee_p)\}/\ln\{1-c(1 - ee_p)\}$, where c is the conversion of the substrate; and ee_p is the enantiomer excess, $ee_p = |[(S)\text{-alcohol}] - [(R)\text{-alcohol}]|/\{[(S)\text{-alcohol}] + [(R)\text{-alcohol}]\} \times 100\%$. In the formulas, the quantity in the square brackets denotes molar concentration.

2.4. Chemical modification of the lipase

The lipase powder adopted in the article was a semi-purified form and contains only *Pc*L protein and some salts. Before modification, the powder was dissolved in deionized water, and added into a centrifuge tube (10 mL) with ultrafiltration membrane (molecular weight cut off = 10 kDa), centrifuged at 12,000 rpm at 4 °C for 5 min to remove the salts. The purified lipase was afterwards dissolved in buffer solutions for modification study. Arg (Arginine residue) was modified by CHD according to Patthy et al. [19]; Asp (aspartic acid residue), Glu (glutamic acid residue) and Lys (lysine residue) was modified by EDA, EDA and TNBS according to Barbosa et al. [20] and Geoghegan [21]; His (histidine residue) was modified by DEPC according to Miles et al. [22]; Met (methionine residue) was modified by IAA according to Gundlach et al. [23]; Trp (tryptophan residue) was modified by HNBB according to Hortan et al. [24]; Tyr (tyrosine residue) was modified by NAI, ICl and TNM according to Cacace et al. [25], Koshland et al. [26] and Riordan et al. [27]. All modification processes were carried out in an ice bath to avoid thermal inactivation. The modified *Pc*L was desalted using an AKTA Prime protein purifier (GE Healthcare, Little Chalfont, Buckinghamshire, UK) equipped with a HiTrap desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

The NAI-modified Tyr can be recovered by hydroxylamine: the reaction conditions were: 1 mL of the desalted *Pc*L was mixed with 30 μL hydroxylamine (50% aqueous solution) and reacted for 1 h. The resulting mixture was vacuum frozen for 12 h.

In addition to the standard reaction conditions of the chemical modification method referred to above, *n*-hexane was employed as an activator of *Pc*L, because lipases are widely known to be activated at the aqueous-hydrophobic interface [28–30]. In the modification reaction coupled with activation, 10% (*V*/*V*) of *n*-hexane was added to the mixture under the same reaction condition.

2.5. Protein mass spectrum

Chemically modified *Pc*L was isolated by SDS-PAGE according to Vandahl et al. [31]. The resulting denatured pure *Pc*L was then digested by trypsin at 37 °C overnight, according to Chen et al. [32]. The digested peptide slices were estimated by ExPASy Peptide-Mass (http://web.expasy.org/peptide_mass/). Finally, the sample was lyophilized using a Labconco FreeZone 18 freeze drier (Labconc Corporation, Kansas City, MO, USA) and afterwards dissolved in 20 μL of acetonitrile with 0.1% trifluoroacetic acid (1: 9, *V*/*V*) for subsequent mass spectrometric analysis according to Zehl et al. [33]. α-Cyano-4-hydroxycinnamic acid was used as the matrix and ions were extracted into the linear UltraFleXtreme MALDI-TOF/TOF spectrometer (Bruker Corporation, Boston, MA, USA) using an extraction potential of 20 kV in the high-mass detection mode according to Chang et al. [34]. Peptide mass fingerprinting data was also employed to search in NCBI non-redundant protein databases using the Mascot software (Matrix Science Inc., Boston, MA, USA).

2.6. Simulation

The XRD (X-ray distraction) crystalline structure of *Pc*L (PDB ID: 2NW6) was used as a starting structure of the modeled lipase. The ligand ((phenoxymethyl)propyl methylphosphonochloridoate, named POT in the file) and crystal water molecules (named HOH in the file) in the PDB file, were manually deleted in the Tripos SYBYL-X 1.1 (Tripos Associates, St. Louis, MO, USA) workspace window. Then a pK_a calculation for the titratable residues of the protein at pH 7 was performed using the online tool PCE [35]. Accordingly, the side-chain carboxyl groups of Asp^{2}, Asp^{21}, Asp^{36}, Asp^{55}, Asp^{56}, Asp^{102}, Asp^{121}, Asp^{130}, Asp^{159}, Asp^{228}, Asp^{236}, Asp^{242}, Asp^{264}, Asp^{288}, Asp^{303}, Glu^{28}, Glu^{35}, Glu^{63}, Glu^{118}, Glu^{197}, Glu^{289} and Glu^{302} were deprotonated and charged with −1.00; phenoxy oxygen atoms (O^{η}) of Tyr^{4}, Tyr^{9}, Tyr^{23}, Tyr^{29}, Tyr^{31}, Tyr^{45}, Tyr^{68}, Tyr^{95}, Tyr^{129}, Tyr^{175}, Tyr^{179}, Tyr^{207}, Tyr^{274} and Tyr^{282} were protonated; His^{15}, His^{86}, His^{114}, His^{204} and His^{311} were set to the HIE state (each with a deprotonated N^{δ} and a protonated N^{ε}); side-chain amino groups of Lys^{22}, Lys^{70}, Lys^{80}, Lys^{165}, Lys^{269}, Lys^{283} and Lys^{316} and guanidyl groups of Arg^{8}, Arg^{40}, Arg^{61}, Arg^{94}, Arg^{115}, Arg^{258}, Arg^{297}, Arg^{309}, Arg^{314} were protonated and charged with +1.00; the N-terminal amino group was protonated and charged with +1.00 and the C-terminal carboxyl group was deprotonated and charged with −1.00. Additionally, the catalytic His^{286} was set to the HIP state (with both N^{δ} and N^{ε} protonated) with a charged state of +1.00, and the O^{γ} of the catalytic Ser^{87} was deprotonated.

The tetrahedral intermediates of the chiral primary alcohol esters' enantiomers were generated by SYBYL-X. Starting with the resulting substrate structure and the enzyme structure prepared above, the substrate-enzyme complex in which acyloxy carbon atom of the tetrahedron covalently bonded to the catalytic group (deprotonated Ser^{87}-O^{γ}) was elucidated by the covalent docking module FlexX of SYBYL-X according to Chen et al. [32]