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Enhancement of methanol resistance of *Yarrowia lipolytica* lipase 2 using β-cyclodextrin as an additive: Insights from experiments and molecular dynamics simulation

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ABSTRACT

The methanol resistance of lipase is a critical parameter in enzymatic biodiesel production. In the present work, the methanol resistance of *Yarrowia lipolytica* Lipase 2 (YLLIP2) was significantly improved using β-cyclodextrin (β-CD) as an additive. According to the results, YLLIP2 with β-CD exhibited approximately 7000 U/mg specific activity in 30 wt% methanol for 60 min compared with no activity without β-CD under the same conditions. Molecular dynamics (MD) simulation results indicated that the β-CD molecules weakened the conformational change of YLLIP2 and maintained a semi-open state of the lid by overcoming the interference caused by methanol molecules. Furthermore, the β-CD molecule could directly stabilize "pathway" regions (e.g., Asp61-Asp67) and indirectly stabilize "pathway" regions (e.g., Gly44-Phe50) by forming hydrogen bonds with "pathway" regions and nearby "pathway" regions, respectively. The regions stabilized by the β-CD molecule then prevented the closure of active pockets, thus retaining the enzymatic activity of YLLIP2 with β-CD in methanol solvent.

1. Introduction

Methanol is mostly used for biodiesel (fatty acid alkyl esters, FAAEs) production given its low cost; thus, FAAEs most commonly refers to fatty acid methyl esters (FAMEs) [1]. Chemical catalysts for production of biodiesel are widely adopted in industry. However, waste water treatment with acids or bases and reuse is a severe problem both from an energy-consuming and environmental point of view. In contrast, enzymes could be used to produce biodiesel in a green manner with respect to less energy consumption and less waste treatment [2].

In our previous work, *Yarrowia lipolytica* Lipase 2 (YLLIP2) was isolated from a mutant strain *Candida* sp. 99–125 (belonging to *Yarrowia lipolytica)* and was employed in biodiesel production [3]. However, the denaturation caused by methanol is the major employment obstacle for lipase [4]. To overcome the negative effect of methanol, stepwise addition was developed to avoid the inactivation caused by polar short-chain alcohols [5]. However, the methanol resistance of lipase did not improve with the use of stepwise addition. Generally, two main approaches are used to obtain desired properties of enzymes: sequence remodelling at the gene level (such as rational design and directed evolution) [6] and structure rearrangement around the enzyme molecule (such as immobilization and solvent engineering) [7,8]. Although sequence remodelling is a direct method to tailor enzyme properties, it is still limited based on the knowledge-cost (structure-function relationship) and time-cost (high-throughput). As a practical and effective method in industry, additives have been employed to improve enzyme properties in reaction systems [9,10].

Molecular dynamics (MD) simulation is a useful tool for understanding protein structure and has been used to offer insights into the structure and behaviour of enzymes in different solvent environments [11–16]. Based on the reported crystal structure of YLLIP2 by Bordes et al. [17], our previous simulation work studied several properties in solvent environments: the activation mechanism of YLLIP2 immobilized on carbon nanotubes (CNTs) [18],

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the effects of temperature and solvent environments on YLLIP2 [19] and the inactivation mechanism of YLLIP2 in methanol solvent [20]. Furthermore, two interaction propagation "pathways", which are crucial for controlling active pocket closing of YLLIP2, were presented to further understand the inactivation mechanism of YLLIP2 in methanol solvent [21]. In the lid closure process of YLLIP2, conformational rearrangement of the Ser274-Asn288 region is the beginning of the interaction propagation. The driving force for the motion and conformational rearrangement is derived from its abundant hydrophobic surface. The Ser274-Asn288 region propagates its interaction through the "pathways" (several protein zones) to the lid region and makes the active pocket close.

β-CD is a doughnut-shaped structure consisting of seven glucose units linked by a 1,4-glycosidic bonds. The outside of β-CD exhibits hydrophilic properties due to the hydroxyl groups, whereas the inside of β-CD exhibits hydrophobic properties due to hydrogen bonds. Based on the complex properties of β-CD, the application containing β-CD for the enzyme-catalysed reaction provides several advantages, including an enhanced reaction rate and product yield and reduced instability of the enzyme caused by the organic solvent [22]. Recently, Nie et al. improved the enzymatic synthesis of biodiesel from waste oil with YLLIP2 using β-CD as an additive in a solvent-free system [23]. The positive effect of β-CD on the bio-catalytic reaction might be complex, such as emulsification, shift of substances and modification of lipase [24–26].

Regarding biodiesel production, the effect of β-CD as an additive on the methanol resistance of YLLIP2 was independently investigated in the present work. The remaining activity of YLLIP2 was assessed in different ratios of methanol-water mixtures corresponding to the practical concentrations of methanol in the biodiesel industry. Furthermore, the remaining activity of YLLIP2 with β-CD in the30 wt% methanol solvent demonstrated that β-CD molecules protect the conformation of the protein against the interference caused by methanol molecules in the extreme condition and provided a view that β-CD could be used as a protective agent in polar-solvent engineering for the lipase-like YLLIP2.

2. Materials and methods

2.1. Reagents

β-CD was of analytical grade and purchased from Ziyi Chemical Reagent Co. (Shang Hai, China). Olive oil and polyvinyl alcohol (PVA) obtained from Sanbo Biotech Co. (Beijing, China) were of chemical grade. The rest of the chemicals were of analytical grade and purchased from Beijing Chemical Factory.

2.2. Enzyme purification

The methods of purification and deglycosylation have been reported by Yu et al. [27]. Purified deglycosylated YLLIP2 was obtained from the crude extract of the mutant strain *Candida* sp. 99–125 (belonging to *Yarrowia lipolytica*) via ion-exchange chromatography and hydrophobic interaction chromatography successively.

2.3. Lipase activity assay

Lipase activity was measured by titrimetric assay according to an olive oil emulsion [27,28]. Olive oil [5% (v/v)] was emulsified in distilled water containing 2% (w/v) of PVA in a homogenizer for 6 min at maximum speed. Then the enzyme solution (1 ml) was added to 5 ml of substrate emulsion and 4 ml of 100 mM phosphate buffer, pH 8.0 (Na_2HPO_4–KH_2PO_4). Lipase activity was determined by titration of the fatty acid released with 50 mM NaOH. The protein content was determined by the Bradford protein assay method using bovine serum albumin as standard [29].

2.4. Methanol tolerance assay

Different concentrations of methanol (10 wt%, 20 wt%, 30 wt%) with lipase were obtained by adding a specific amount of methanol into 200 ml of purified YLLIP2 (0.1 mg/ml protein in 100 mM phosphate buffer, pH 7.0) in a conical flask. β-CD was added into the reaction system (β-CD:lipase = 1:1, w/w) and the flasks were incubated in a water bath at 30 °C with a stirring of 100 rpm 1.0 ml of the reaction mixture was taken at 0 min, 10 min, 30 min, 60 min and 120 min. Each sample was flushed with nitrogen to remove methanol and was then prepared through freeze-drying process. The lipase activity was subsequently measured at 37 °C. The control experiments without β-CD were performed and the lipase activity was also assayed.

2.5. Protein structure in simulation

An open conformation of YLLIP2 was used as the initial conformation of our study. Since there was no open conformation of YLLIP2 available in the Protein Data Bank (PDB), we obtained the open conformation after immobilizing its closed conformation (PDB ID: 3O0D) on carbon nanotubes (CNTs) in our previous work [18]. This open conformation was examined to be suitable to study the features of activated YLLIP2 [20,21]. The CNTs molecules were striped and the water molecules around YLLIP2 within 0.5 nm were retained during all the simulation processes.

2.6. Molecular dynamics simulation

All molecular dynamics (MD) simulations were performed using the AMBER suite [30–33]. The all-atom ff03 force field [34–36] was used to model the lipase and the GLYCAM-06 force field [37] was used for β-CD. The protein YLLIP2 was evenly surrounded by 11 β-CD molecules and then embedded in a periodic rectangular parallelepiped solvent box containing 9248 water molecules and 2001 methanol molecules (approximately 30 wt%). The control group without β-CD molecules was constructed using the same solvent system. The solvent molecules left a 10 Å space around the protein. Several Na^+ cations were added to neutralize the systems.

An energy minimization was performed to eliminate improper contacts in each system. We used the steepest descent method in the first 5000 steps and the conjugate gradient method in the last 5000 steps. After the energy minimization, each system was heated gradually from 0 to 298 K. During the heating steps, position restraints were imposed on the lipase with a force constant of 10.0 kcal/mol/$Å^2$. Each system was then equilibrated for 500 ps at constant temperature (298 K) and pressure (1 bar) conditions via the Langevin dynamics (the collision frequency is 1.0 ps^{-1}), with a coupling constant of 0.2 ps for both parameters. The production simulation was performed for 100 ns at 298 K and 1 bar using a time step of 2 fs. Electrostatic interactions were calculated using the particle-mesh-Ewald algorithm. The cut-off distance for van der Waals interactions was 10.0 Å. The SHAKE algorithm [38] was applied to all bonds involving hydrogen.

2.7. Analytical methods

To reduce the bias of the initial atom velocities and position of β-CD molecules, three independent 100-ns MD simulations with different β-CD distributions and different starting atomic velocities were performed. For each independent MD run, the interacted regions between β-CD molecules and YLLIP2 are conservative at 100 ns. The patterns of the RMSd of backbone atoms, the distance