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Computational approach for designing thermostable *Candida antarctica* lipase B by molecular dynamics simulation

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ABSTRACT

Candida antarctica lipase B (CalB) is one of the most useful enzyme for various reactions and bioconversions. Enhancing thermostability of CalB is required for industrial applications. In this study, we propose a computational design strategy to improve the thermostability of CalB. Molecular dynamics simulations at various temperatures were used to investigate the common fluctuation sites in CalB, which are considered to be thermally weak points. The RosettaDesign algorithm was used to design the selected residues. The redesigned CalB was simulated to verify both the enhancement of intramolecular interactions and the lowering of the overall root-mean-square deviation (RMSD) values. The A251E mutant designed using this strategy showed a 2.5-fold higher thermostability than the wild-type CalB. This strategy could apply to other industry applicable enzymes.

1. Introduction

Biotechnology research toward improving the thermostability of enzymes is important for potential benefits in industrial applications (Fagain, 1995). Numerous strategies have been developed to improve the thermostability of proteins (Bornscheuer and Pohl, 2001; Eijsink et al., 2005; Pack and Yoo, 2004; Steipe, 1999). Rational design approaches based on a detailed understanding of enzyme structure and function have identified important factors that affect the thermostability of enzymes (Böttcher and Bornscheuer, 2010; Kim et al., 2010a,b). Lowering the overall RMSD is one way to modify the enzyme structure to make it more rigid, which could enhance the thermostability. Generally, thermophilic enzymes are more rigid, more stable and show lower RMSD values than mesophile enzymes. Common stabilizing strategies, such as introducing disulfide bonds or salt bridges, also result in lower RMSD values. In addition, enhancing intramolecular interactions, such as ionic interactions or hydrophobic interactions, also leads to a decrease in the overall RMSD value of an enzyme (Chen et al., 2013; Colombo and Merz, 1999; Noorbatcha et al., 2012). It is thus, lowering the overall RMSD is an important factor for enhancing enzyme thermostability. However, there is still no efficient strategy for finding the weak-point residues of an enzyme for modulation. Molecular dynamics (MD) simulation has been shown to be a useful tool for understanding enzyme structure and behavior. Using MD simulations, it is possible to observe different enzyme motions at various temperatures. MD simulations at various temperatures are necessary to not only predict the weak point of the enzyme but also to verify enzyme rigidity.

Candida antarctica lipase B (CalB) is an efficient biocatalyst for hydrolysis in water and for the esterification in organic solvents (Anderson et al., 1998). This catalyst is used in numerous industrial applications due to its high enantioselectivity, wide range of substrates and thermal stability. In particular, biodiesel production using CalB has drawn much attention due to the high activity and stability of CalB in the presence of alcohols. These processes are normally operated at high temperatures, and thus improving the thermostability of CalB is desirable. Majority MD simulations of CalB were reported for understanding the mechanism and movements (Gruber and Pleiss, 2012; Park et al., 2013), thus simulations for engineering purpose are required.

In this study, a rational design strategy was developed to improve the thermostability of CalB. MD simulations at different temperatures were performed to predict common fluctuation residues, which were then considered to be thermally weak-point

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Table 1
Mutation primers and the specific activity of the mutants.

Enzymes	Mutation primers	Relative activity (%)
[a]Wild-type		100
A146D	Forward: 5′ CCTCGATGACCTCGCGGTTAGTGCACCCTC 3′ Reverse: 5′ CTAACCGCGAGGTCATCGAGGGGGCCGGCG 3′	87
T158S	Forward: 5′ GGCAGCAATCCACCGGTTCGGCACTCACC 3′ Reverse: 5′ CGAACCGGTGGATTGCTGCCATACGGAGG 3′	209
A251E	Forward: 5′ CTCGTAGTGAGGACTACGGAATCACGG 3′ Reverse: 3′ CGTAGTCCTCACTACGAGCCTGGCCCG 5′	50

[a]Wild-type enzyme specific activity was 1.65 U/mg enzyme.

residues. After analyzing the simulation results, computational design with increased intramolecular interactions and lowered overall RMSD were applied to the target residues. This developed strategy successfully designed a thermostable CalB for industrial application.

2. Materials and methods

2.1. Materials

The gene encoding the lipase CalB was kindly donated by Prof. Yong Hwan Kim at Kwangwoon University. Restriction enzymes were purchased from Enzynomics (Dajeon, Korea). The *Pichia pastoris* strain X-33 and the *pPICZalpha*A plasmid were purchased from Invitrogen (Carlsbad, USA). Para-nitrophenyl caprylate (pNPC) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All PCR primers were synthesized at COSMO GENTECH (Seoul, Korea).

2.2. Gene cloning and recombinant expression of CalB variants

The CalB gene was subcloned into the *pPICZalpha*A plasmid using *Xho*I and *Xba*rI. Site-directed mutagenesis of the CalB gene was performed according to the QuickChange™ protocol. All primers used for mutagenesis are listed in Table 1. The *pPICZalpha*A-CalB genes were then transformed into *P. pastoris* X-33 using the lithium chloride method. Positive transformants were inoculated into 25 ml of buffered glycerol-complex medium (BMGY), which consisted of a 100 mM potassium phosphate buffer (pH 6.0) with 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, and 1% glycerol in a 25 ml baffled flask. The cells were cultured at 29 °C with shaking at 250 rpm in an incubator until the culture reached an OD_{600} of 2–6. The cells were subsequently harvested by centrifugation at 1500–3000 × *g* for 5 min. The supernatant was decanted, and the cell pellet was resuspended to an OD_{600} of 1.0 in a buffered-methanol complex medium (BMMY), which consisted of a 100 mM potassium phosphate buffer (pH 6.0) with 1% yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol to induce expression. Methanol (100%) was added to a final concentration of 0.5% every 24 h to maintain the induction. After 5 days, the supernatant containing the secreted CalB variants was obtained by centrifugation at 3800 rpm at 4 °C for 30 min.

2.3. Enzyme purification by FPLC

The supernatant containing the enzyme was concentrated using an Amicon Ultra membrane (MWCO = 30 kDa). Ion-exchange chromatography was conducted by FPLC. The crude solution of CalB was purified using cation-exchange chromatography. An XK 16/20 column (Amersham Biosciences) filled with 7 ml Source 15S resin was equilibrated. All chromatography experiments were performed at room temperature using an AKTA explorer chromatography system controlled by Unicorn software. A more detailed version of this protocol has been previously published (Trodler et al., 2008).

2.4. Activity assay and stability measurements

pNPC was used to compare the activity of the CalB variants. pNPC was dissolved in acetonitrile to 33.3 mM and mixed with a reaction buffer (50 mM sodium phosphate buffer at pH 8.0 with 0.5% Triton X) at a ratio of 1:9. The enzyme concentration used was 75 μg/ml. The final composition of this reaction solution was 100 μl substrate, 900 μl reaction buffer, and 20 μl enzyme. The reaction solution was then vortexed moderately. The para-nitrophenol (pNP) release rate was determined by measuring changes in absorbance at 405 nm for 10 min at 30 °C, as measured with a Cary 50 UV spectrophotometer (Varian, CA, USA). The molar extinction coefficient of pNP in the reaction was 16,935 M^{-1} cm^{-1}.

The thermostability was determined by measuring the residual activity of CalB after incubation at 40, 50, or 60 °C for 1 h. The long-term residual activity in 50 °C was measured every 60 min for 4 h. All experimental data shown are the averages of triplicate measurements.

2.5. Computational design of CalB and mutants

The crystal structure of CalB (PDB code: 1TCA) (Uppenberg et al., 1994) was used for the simulations. The RosettaDesign web server (http://rosettadesign.med.unc.edu) was used to predict the target residue mutation. The *in silico* design of the mutants was created using the Discovery Studio 2.5.5 package with the mutation information obtained by RosettaDesign (Hu and Kuhlman, 2006). The minimization of structures was preceded by 1000 steps of the steepest descent method and another 1000 steps of the Newton Raphson method. The CHARMm force field and the Momany–Rone partial charge were used to assign atom types. The crystallographic water molecules were retained to mimic a hydration shell. The solvent-accessible surface area was calculated using the GetArea web-based server (http://curie.utmb.edu/getarea.html) (Fraczkiewicz and Braun, 1998). The intramolecular interactions were calculated using the PIC server (Tina et al., 2007).

2.6. Molecular dynamics simulation

The MD simulations of CalB were performed using the Discovery Studio 2.5.5 package. Simulations of the enzyme structures were performed using the Standard Dynamics Cascade protocol. All simulations were performed according to the following parameters. The maximum steps of the steepest descent method and the Newton Raphson method were set to 2000. The equilibration and production steps were 200 ps and 1 ns, respectively. In the production step, production temperatures were 300 K, 330 K, 360 K, and 400 K (for the mutants, only 360 K was simulated for verification). The trajectory of the BCX and CalB were saved every 50 ps, and 20 trajectories were analyzed for comparison.

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