Electronic Supplementary Material (ESI) for RSC Advances


# Electronic Supplementary Information

# Rational design for enhancing promiscuous activity of *Candida antarctica* lipase B: a clue for molecular basis of dissimilar activities between lipase and serine-protease

Suhyun Jung, Juhyun Kim and Seongsoon Park*

## Experimental Section

### Materials and Method

Chemicals and buffers were purchased from Sigma-Aldrich. *Pfu* DNA polymerase and restriction enzyme (*Dpn* I) were purchased from Enzynomics (Daejeon, Korea) and New England Biolabs, respectively. DNA oligomers were obtained from Sigma-Proligo (Singapore). DNA sequencing was performed by Solgent Co. (Daejeon, Korea). The Ni-NTA agarose resin was purchased from QIAGEN.

### Acylation of 1-phenylethanol and 1-phenylethanamine

Acyl donor reagent (1.0 mmol) and 1-phenylethanol or 1-phenylethanamine (1.0 mmol) were added to a suspension of molecular sieves 4A (50 mg) and Novozym 435 (10 mg) in *tert*-butylmethylether (5.0 ml) and shaken at 20 °C. The samples (100 μl) from the reaction mixture were retrieved with intervals and analyzed by a GC with a chiral column (Cyclosil-B, 30 m × 0.25 mm): initial column temperature 80 °C for 10 min, ramp to 120 °C at a rate of 2.5 °C $min^{-1}$, and then held at 120 °C for 10 min.

**Site directed mutagenesis**

The mutants were prepared by the QuikChange mutagenesis kit (Stratagene) using the following forward and reverse primers (Table S1). The plasmids of the mutants were transformed into *E. coli* (Top10).

***Table S1***. The primers used for mutagenesis

| | |
|---|---|
| F_mu_CALB_G39M | 5'-CCGATCCTGGTACCGATGACCCGTACCACTGGC-3' |
| R_mu_CALB_G39M | 5'-GCCAGTGGTACGGGTCATCGGTACCAGGATCGG-3' |
| F_mu_CALB_G39D | 5'-AAACCGATCCTGGTACCGATGACCCGTACCACTGGC-3' |
| R_mu_CALB_G39D | 5'-GCCAGTGGTACGGGTCATCGGTACCAGGATCGGTTT-3' |
| F_mu_CALB_G39E | 5'-AAACCGATCCTGCTGGTACCGGAAACCGGTACCACTGGC-3' |
| R_mu_CALB_G39E | 5'-GCCAGTGGTACCGGTTTCCGGTACCAGCAGGATCGGTTT-3' |
| F_mu_CALB_G281M | 5'-GCGGCTCTGGCACCGATGGCAGCTGCAATTGTT-3' |
| R_mu_CALB_G281M | 5'-AACAATTGCAGCTGCCATCGGTGCCAGAGCCGC-3' |
| F_mu_CALB_G281D | 5'-GCGGCTCTGGCACCGGATGCAGCTGCAATTGTT-3' |
| R_mu_CALB_G281D | 5'-AACAATTGCAGCTGCATCCGGTGCCAGAGCCGC-3' |
| F_mu_CALB_G281E | 5'-GCGGCTCTGGCACCGGAAGCAGCTGCAATTGTT-3' |
| R_mu_CALB_G281E | 5'-AACAATTGCAGCTGCTTCCGGTGCCAGAGCCGC-3' |
| F_mu_CALB_I189M | 5'-AGCGCAACCGATGAGATGGTTCAGCCGCAGGTATC-3' |
| R_mu_CALB_I189M | 5'-GATACCTGCGGCTGAACCATCTCATCGGTTGCGCT-3' |
| F_mu_CALB_I189D | 5'-AGCGCAACCGATGAGGACGTTCAGCCGCAGGTATC-3' |
| R_mu_CALB_I189D | 5'-GATACCTGCGGCTGAACGTCCTCATCGGTTGCGCT-3' |
| F_mu_CALB_I189E | 5'-CAGCGCAACCGATGAGGAAGTTCAGCCGCAGGTATCTAAC-3' |
| R_mu_CALB_I189E | 5'-GTTAGATACCTGCGGCTGAACTTCCTCATCGGTTGCGCTG-3' |
| F_mu_CALB_I189Q | 5' GCGCAACCGATGAGCAGGTTCAGCCGCAG-3' |
| R_mu_CALB_I189Q | 5' CTGCGGCTGAACCTGCTCATCGGTTGCGC-3' |

**Expression and purification of CAL-B**

Overnight culture (1 ml of *E. coli*) was added to LB medium (100 ml; ampicillin, 100 µg/ml) and incubated at 37 °C and 200 rpm to an $OD_{600}$ of 0.5. Protein expression was induced by adding arabinose (1 ml; 2% w/v) and incubated for 6 h at 25 °C and 200 rpm. The $OD_{600}$ was reached to ~1.5. The cells were harvested by centrifugation (10 min, 3,800 × g, 4 °C) and the supernatant was discarded. The cell pellet (~0.8 g) was resuspended in the lysis buffer (5 ml/g wet weight; $NaH_2PO_4$, 50 mM; NaCl, 300 mM; imidazole, 10 mM; adjusted to pH 8.0 with NaOH), and lysozyme was added to 1 mg/ml. Incubation on ice for 45 min was followed by a freeze-thaw cycle at -20 °C and room temperature. The viscous lysate was passed several times through a sterile 20-gauge syringe needle and centrifuged (10 min, 10,000 × g, 4 °C). The supernatant was separated from the cell debris. The cell debris was dissolved in 8M urea solution (4 ml, containing 1 mM of dithiothreitol) for SDS-PAGE analysis. Ni-NTA agarose resin (1 ml, 50% w/v slurry) was added to the supernatant (4 ml) and the mixture was stirred at 25 °C for 1 h. The lysate-Ni-NTA mixture was loaded on a Poly-Prep column (Bio-Rad), drained, and then washed three times with the wash buffer (4 ml; $NaH_2PO_4$, 50 mM; NaCl, 300 mM; imidazole, 20 mM; adjusted to pH 8.0 with NaOH). The $His_6$-CAL-B enzyme was eluted from the column with four volumes of the elution buffer (0.5 ml; $NaH_2PO_4$, 50 mM; NaCl, 300 mM; imidazole, 250 mM; adjusted to pH 8.0 with NaOH). Eluate (2 ml) from the Ni-NTA column containing the purified CAL-B was exchanged from the elution buffer to BES (5 mM, pH 7.2) using a centrifugal device (Amicon Ultra-15, Millipore).

**Determination of the amount of the enzymes**

The amount of purified enzymes was determined by absorbance at 280 nm ($\varepsilon$ = 41,285 $M^{-1}$ $cm^{-1}$ calculated with tools at Swiss Prot Expasy, http://ca.expasy.org/tools/protparam.html).

**Measurement of esterase activity toward hydrolysis of 4-nitrophenylacetate**

Esterase activity of the enzymes was measured by following the hydrolysis of 4-nitrophenylacetate at 405 nm. The assay solution was prepared by mixing 4-nitrophenylacetate (20 µl, 200 mM in acetonitrile), acetonitrile (870 µl), and BES buffer (5 mM, pH 7.2, 11,110 µl). The absorbance change was measured at 405 nm for 5 min after mixing the assay solution (100 µl) with the enzyme solution (5 µl). The final concentrations in the reaction solution were 0.32 mM substrate, 4.65 mM BES, 7% acetonitrile. The activity was calculated according to the method of Janes et al. (*S1*) where $\Delta\varepsilon$ = 17,300 $M^{-1}$ $cm^{-1}$.

**Measurement of amidase activity toward hydrolysis of 4-nitroacetanilide**

Amidase activity of the enzymes was measured from the hydrolysis of 4-nitroacetanilide at 405 nm. The assay solution was prepared by mixing 4-nitroacetanilide (200 µl, 100 mM in acetonitrile), acetonitrile (690 µl), and BES buffer (11,110 µl , 5 mM, pH 7.2). The assay solution (1 ml) and the enzyme solution (100 µl) were combined and incubated at 24 °C. The final concentrations in the reaction solution were 1.52 mM substrate, 4.66 mM BES, 7% acetonitrile. The enzyme concentrations used were 1.5-2.6 µM. The absorbance change was measured at 405 nm. The activity was calculated using $\Delta\varepsilon$ = 11,100 $M^{-1}$ $cm^{-1}$.

**HPLC analyses**

The samples (500 μl) conducted with the hydrolysis of 4-nitroacetanilide were diluted with acetonitrile (500 μl). The diluted samples (1 μl) were injected on a nonpolar column (3.0 × 100 mm, 1.8-μm thickness, Agilent ZORBAX Eclipse plus C18), and eluted with an aqueous methanol solution (60% v/v) containing 0.2% of acetic acid at a flow rate of 0.2 ml $min^{-1}$ and 25 °C. The signals were detected at 320 nm. The retention times of 4-nitroacetanilide and 4-nitroaniline were 3.4 min and 4.1 min, respectively.

**Molecular modeling**

Computer modeling was performed using the SYBYL 8.1 package (Tripos). The X-ray crystal structure of CAL-B (pdb entry 1TCA) was obtained from the Protein Data Bank. The 189 residue was substituted with glutamine. The enzyme structure containing the tetrahedral intermediate form of the substrate was manually created in accordance with the previous literature (*S2*). The AMBER99 force field (*S3*) was used with a distant dependent dielectric constant and a nonbonded cut-off of 8 Å. The atom types and parameters undefined by the AMBER99 force field of the substrate were assigned using the general AMBER force field (GAFF) (*S4*). Partial charges of the atoms of the enzyme and the tetrahedral form of the substrate were calculated by the AMBER method and the Gasteiger-Huckel method, respectively, with a formal charge of -1 for the substrate oxyanion. Energy-minimization calculation was performed by the Powell method in the SYBYL program and proceeded until the rms derivatives reached less than 0.005 $kcal \cdot mol^{-1} \cdot Å^{-1}$. The models were visualized using PYMOL (http://www.pymol.org).

## Supporting Table

**Table S12. Initial rates of acylation of 1-phenylethanamine by Novozym 435** [a]

| Acyl donor | Initial rate [a] (μmol $min^{-1}$ $mg^{-1}$) | Relative rate |
|---|---|---|
| Methyl butyrate | 0.016 ± 0.0012 | 1 |
| Methyl methoxyacetate | 1.56 ± 0.049 | 96 |
| Methyl methylthioacetate | 0.11 ± 0.0068 | 6.8 |

[a] The enantiomeric ratio (*E*) is higher than 200 in all the cases.
[b] Errors were calculated from at least three measurements.

## Supporting Figures

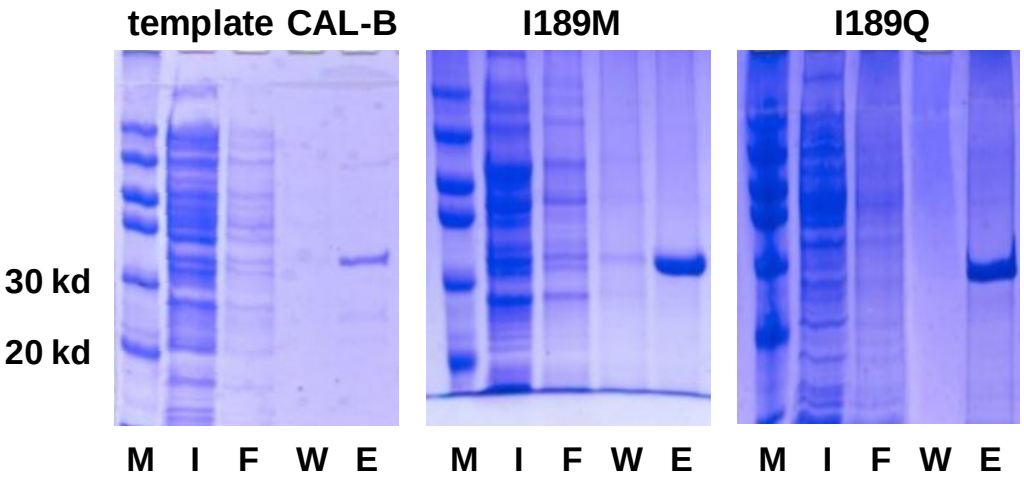


***Fig. S1*** SDS PAGE analyses of CAL-B mutant enzymes expressed in *E. coli*. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; I, insoluble fraction; F, flow-through fraction; W, wash buffer fraction; E, elution buffer fraction (for details see experimental section). The molecular weight of CAL-B is approximately 33 kD.

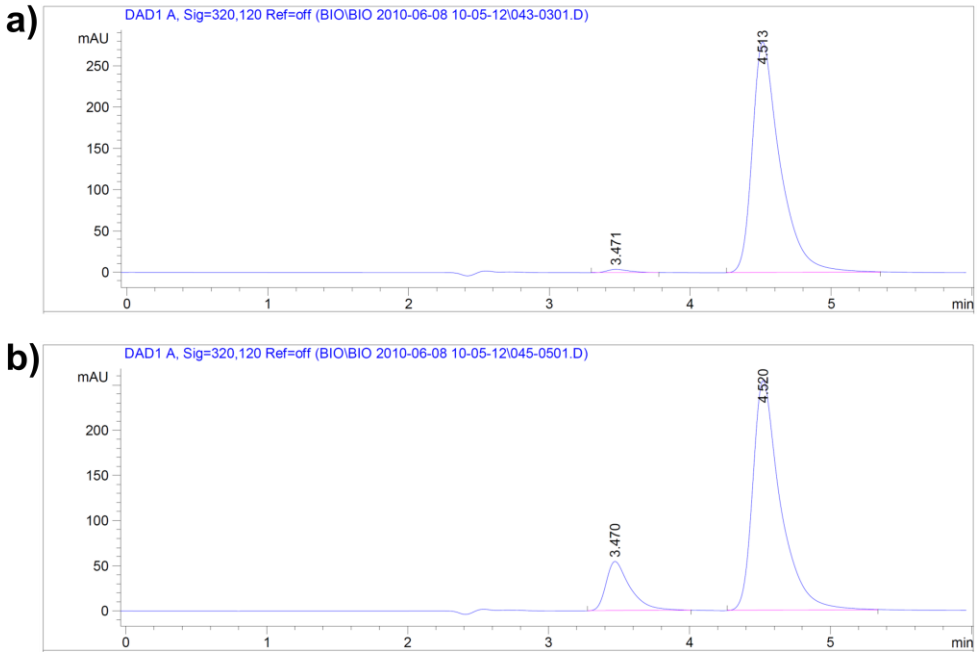


***Fig. S2*** HPLC analyses of the hydrolysis of 4-nitroacetanilide by the CAL-B template and I189Q mutant enzymes. (a) the reaction by the CAL-B template enzyme. (b) the reaction by the I189Q mutant enzyme. 4-Nitroaniline (the product) and 4-nitroacetanilide (the starting compound) were shown at 3.47 min and 4.52 min, respectively. The reaction by the I189Q mutant enzyme clearly produced more products after 48 h. The reaction condition is same to that in Figure 3.

**Supporting References**

*S1*. Janes, L. E.; Cimpoia, A.; Kazlauskas, R. J. *J. Org. Chem*. **1999**, *64*, 9019-9029.

*S2*. Raza, S.; Fransson, L.; Hult, K. *Protein Science*, **2001**, *10*, 329-338.

*S3*. Wang, J.; Cieplak, P.; Kollman, P. A. *J. Comput. Chem.* **2000**, *21*, 1049-1074.

*S4*. Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. *J. Mol. Graph. Model.* **2006**, *25*, 247-260.