Electronic Supplementary Material (ESI) for Chemical Communications

Supporting information

Molecular modeling

Molecular dynamics simulations were performed using the YASARA software.[1] The crystal structure of CALB, 1TCA, was retrieved from the Protein Data Bank. Hydrogen atoms were added to the structure and water molecules were removed from the active site followed by a series of minimization as described by Raza et al.[2] The tetrahedral intermediate was built onto the serine 105 in the active site. A simulation cell with periodic boundaries was set to extend 10 Å at each side of the enzyme. The structure was energy-minimized with the AMBER99 force field[3] with a 7.86-Å force cut-off point and the particle mesh Ewald algorithm[4] to treat long-range electrostatic interactions. Molecular dynamics simulations of 200 ps were performed at 298 K followed by energy minimization and the final structures were used for the prediction of mutants.

Mutagenesis

Site-directed mutagenesis was performed using the pGAPZαB CALB construct[5] as template DNA to make the mutants Q157A, I189A and L278A. Construct pGAPZαB CALB-L278A was used as template DNA to make the double mutant (I189A, L278A). Construct pGAPZαB CALB-I189A, L278A was used as template DNA to make the triple mutant (Q157A, I189A, L278A). The forward primers to mutate portions 157, 189 and 278 were: F-CALB-Q157A (5′-CCC TCC GTA TGG CAG GCA ACC ACA GGT TCG GC-3′), F-CALB-I189A (5′-CGA CCG ACG AGG CCG TTC AGC CTC AGG-3′) and F-CALB-L278A (5′-CGC CGC GGC TGC GCT CGC TGC GCC G-3′). The reverse primers to mutate portions 157, 189 and 278 were: R-CALB- Q157A (5′-GCC GAA CCT GTG GTT GCC TGC CAT ACG GAG GG-3′), R-CALB-I189A (5′-CCT GAG GCT GAA CGG CCT CGT CGG TCG-3′) and R-CALB-L278A (5′-GCA GCT GCC GGC GCA GCG AGC GCA GCC-3′). For the PCR reactions we used: 50 ng of template, 10 pmoles of both forward and reverse primers, 1U Phusion hot start High-Fidelity DNA polymerase, 1x Phusion HF buffer, 0.4 mM dNTP and 3 µL DMSO in total volume of 50 µL. PCR reaction conditions used for making mutants Q157A, I189A, L278A and (I189A, L278A) were; 95 °C for 2 min, 35 cycles with 15 s at 95 °C, 30 s at 55 °C and 6 min 15 s at 68 °C and for making mutant (Q157A, I189A, L278A) were; 98 °C for 60 s, 25 cycles with 10 s at 98 °C, 50 s at 50 °C and 3 min at 72 °C. The DNA sequence of the mutated gene was verified by DNA sequencing.

The PCR reaction mixture was digested with DpnI and 16 µL of the mutagenesis reaction mixture was transformed into the *E. coli* strain XL10-Gold, which was used for the cloning work and the transformants were selected on LB_{Zeo} agar plates. Plasmids were isolated from this construct and linearised with XmaJI (AvrII) (Fast Digest) and electroporated into freshly made electro-competent *P. pastoris* cells SMD1168H (prepared following Invitrogens protocol[6]). Transformed cells were selected on $YPDS_{Zeo}$ plates, incubated at 30 °C until colonies appeared.

Protein expression and activity towards tributyrin

A single cell colony from a fresh YPD_{Zeo} plate was used to inoculate a 10 mL BMGY.[6] This pre-culture was incubated overnight at 30 ºC with an orbital agitation of 260 rpm. Of this culture 0.1 mL was used to inoculate of 100 mL of BMGY. An additional 2 g of glycerol was added after 48 and 72 hours of cultivation. After 96 hours the cells were removed by centrifugation at 1500 x *g* for 10 min at 4 ºC and the supernatant was run through HIC (Hydrophobic Interaction Chromatography) using 5 mL HiTrap Butyl FF HIC (GE Healthcare) prepacked column. Wild-type CALB and mutants Q157A and I189A were eluted at 100 % H_2O while mutants with alanine at position 278 were found to elute at 25 % phosphate buffer (50 mM with 0.8 M ammonium acetate pH 6) 75 % H_2O. A pH-stat (TIM900 Titration Manager, Radiometer, Denmark) was used to measure the hydrolytic activity, by titration of released fatty acid with a 20 mM sodium hydroxide solution. The reaction was started by the addition of enzyme to an emulsion of 0.2 M tributyrin in 2% gum arabicum and 0.2 M $CaCl_2$. The reaction was carried out at room temperature at pH 7.5. The substrate was emulsified by 2 min sonication (Branson sonifier 250) before the addition of enzyme solution.

Immobilization and active site titration

Protein expression similar to what has been described in the previous paragraph was done in 1 l scale. After centrifugation, the supernatant was filtered using centrifugal filter device, Centricon Plus-70 from Millipore. The concentrated protein solutions were diluted in Na^+ phosphate buffer (pH 7, 20 mM) before being immobilized on Accurel enzyme carrier beads as described by Hagström et al.[7] The amount of enzyme loaded on the beads was analyzed by active site titration using the inhibitor methyl 4-methylumbelliferyl hexylphosphonate as described by Magnusson et al.[8] Analysis of inhibition was performed by fluorescence using a Perkin Elmer LS 50 B

fluorimeter. Remaining enzyme activity was measured after inhibition for the WT and all the mutants using a solution contenting 1-hexanol (15 mM), decane (10 mM) and ethyl octanoate (0.5 M) dissolved in cyclohexane. Reaction solution (6 mL) was added to the inhibited enzyme (10 mg) for determination of the degree of inhibition. 5 samples were taken from the reaction at different time intervals and analyzed by gas chromatography (GC). For comparison, the reactions were repeated with the enzymes that had not been subjected to inhibition. The concentration of the immobilized enzyme on the beads were as follow in (w/w %): WT 0.76 %, Q157A 0.084 %, I189A 0.66 %, L278A 0.52 %, (I189A, L278A) 1.5 % and (Q157A, I189A, L278A) 0.065 %.

Kinetics measurements

For studying the kinetics of ROP of *D*,*D*-lactide reactions were run with the WT, Q157A mutant and Q157A, I189A, L278A mutant. In each of the reactions 285 mg (0.8 M) of *D*,*D*-lactide were mixed with 33 mg (0.1 M) of 1-phenylethanol and D_8-toluene was added to set the total volume at 2.5 mL and reaction temperature was 60 ºC. The addition of the enzyme started the reactions and samples of 150 µL were taken after 0.5, 1, 1.5, 2, 3, 4, 5, 6, 24 and 48 hours. The samples were filtered through a piece of cotton in a Pasteur pipette and were added to 400 µL of D_8-toluene and analyzed by ^{1}H NMR without any further purification. For the calculation of the rate of the initiation step, the peak at 4.97 ppm (b in Figure 1S) was used. For the calculation of the rate of the propagation step, the peak at 5.07 ppm (a in Figure 1S) was used.

For studying the kinetics of the transacylation activity towards ethyl octanoate with 1-hexanol we used a solution contenting 1-hexanol (15 mM), decane (10mM) and ethyl octanoate (0.5 M) dissolved in cyclohexane in a total volume of 6 mL. The addition of 10 mg of enzyme started the reaction and 5 samples were taken from the reaction at different time intervals and analyzed by gas chromatography (GC).

Synthesis of poly *D*,*D*-lactide

D,*D*-lactide (285 mg; 2 mmol) was mixed with 32 mg (0.26 mmol) of 1-phenylethanol and D_8-toluene was added to set the total volume at 2.5 mL. The molar ratio between initiator and monomer was thus 1:8. The reactions were started by the addition of 10 mg, 56 mg and 72 mg of

WT, (Q157A) and (Q157A, I189A, L278A) respectively and kept at 60 ºC. The reactions were run for 48 hours and were stopped by filtering off the enzyme.

^{1}H NMR (500 MHz, D_8-toluene, δ in ppm) of poly(*D*,*D*-lactide) initiated with 1-phenylethanol:

5.07 ppm (H, m, -CH(CH_3)OC(O)C*H*(CH_3)O-), 4.79 ppm (H, m, -CH_2CH_2OC(O)C*H*(CH_3)O-), 4.16 ppm (H, m, -C*H*(CH_3)OH), 4.08 ppm (2H, m, Ph-$CH_2C\mathit{H}_2$OC(O)-), 2.61 ppm (2H, m, Ph-CH_2CH_2OC(O)-), 3.88 ppm (2H , t, -OC(O)C*H*(CH_3)O-, free lactide).

Materials

D,*D*-lactide was a generous gift from Purac (Netherlands) and it was run through a Silica column before use in order to remove traces of dilactate. All other chemicals were purchased from Aldrich (Germany) and used as received**.**

Instrumentation

^{1}H NMR spectra of poly *D*,*D*-lactide was recorded on a Bruker AM 500.

MALDI-TOF-MS analyses were conducted on a Bruker UltraFlex MALDI-TOF-MS with SCOUT-MTP Ion Source (Bruker Daltonics, Bremen) equipped with a N_2 laser (337 nm), a gridless ion source and reflector design. All spectra were acquired using a reflector-positive method with an acceleration voltage of 25 and a reflector voltage of 26.3 kV. Calibration was performed in order to secure good mass accuracy. As for the samples, solutions of 2-5 mM in $CHCl_3$ were prepared. The matrix utilized was 9-nitroanthrazene. Matrix solutions were prepared as 0.1 M solutions in THF. The samples were prepared as sample-matrix-Na solutions, employing a 0.1 M of sodium trifluoroacetate solution in THF. The preparation protocol included mixing of 5 μL of sample with 20 μL of matrix. Then 1 μL of the mixture was spotted on the MALDI target and was left to crystallize at room temperature while THF was evaporated. Normally, 50 pulses were acquired for each sample. In order to achieve good mass accuracy and resolution, the analyses were performed at the laser threshold of each individual matrix/sample combination.

Size exclusion chromatography (SEC) using THF as the mobile phase was performed at 35 °C using a Viscotek TDA model 301 equipped with two GMH_{HR}-M columns with TSK-gel (mixed bed, M_W resolving range: 300-100 000) from Tosoh Biosep, a VE 5200 GPC autosampler, a VE 1121 GPC solvent pump, and a VE 5710 GPC degasser (all from Viscotek Corp.). A universal calibration method was created using broad and narrow linear polystyrene standards. Corrections for the flow rate fluctuations were made using toluene as an internal standard. Viscotek OmniSEC version 4.0 software was used to process data.

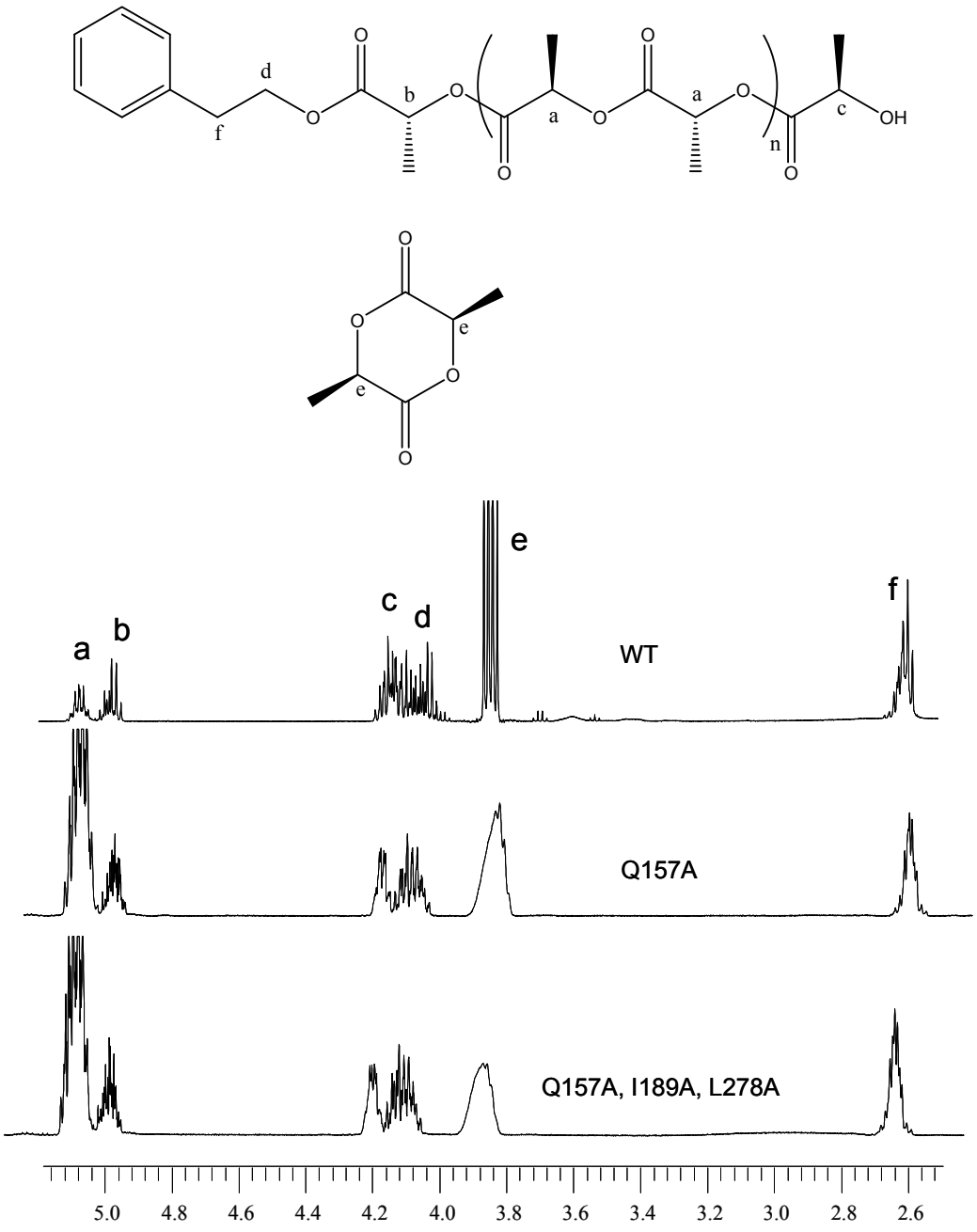

Figure 1S: ^{1}H NMR spectra of the samples at taken after 24 hours of the kinetic reaction of RO P of *D*,*D*-lactide using WT and mutants Q157A and Q157A, I189A, L278A.

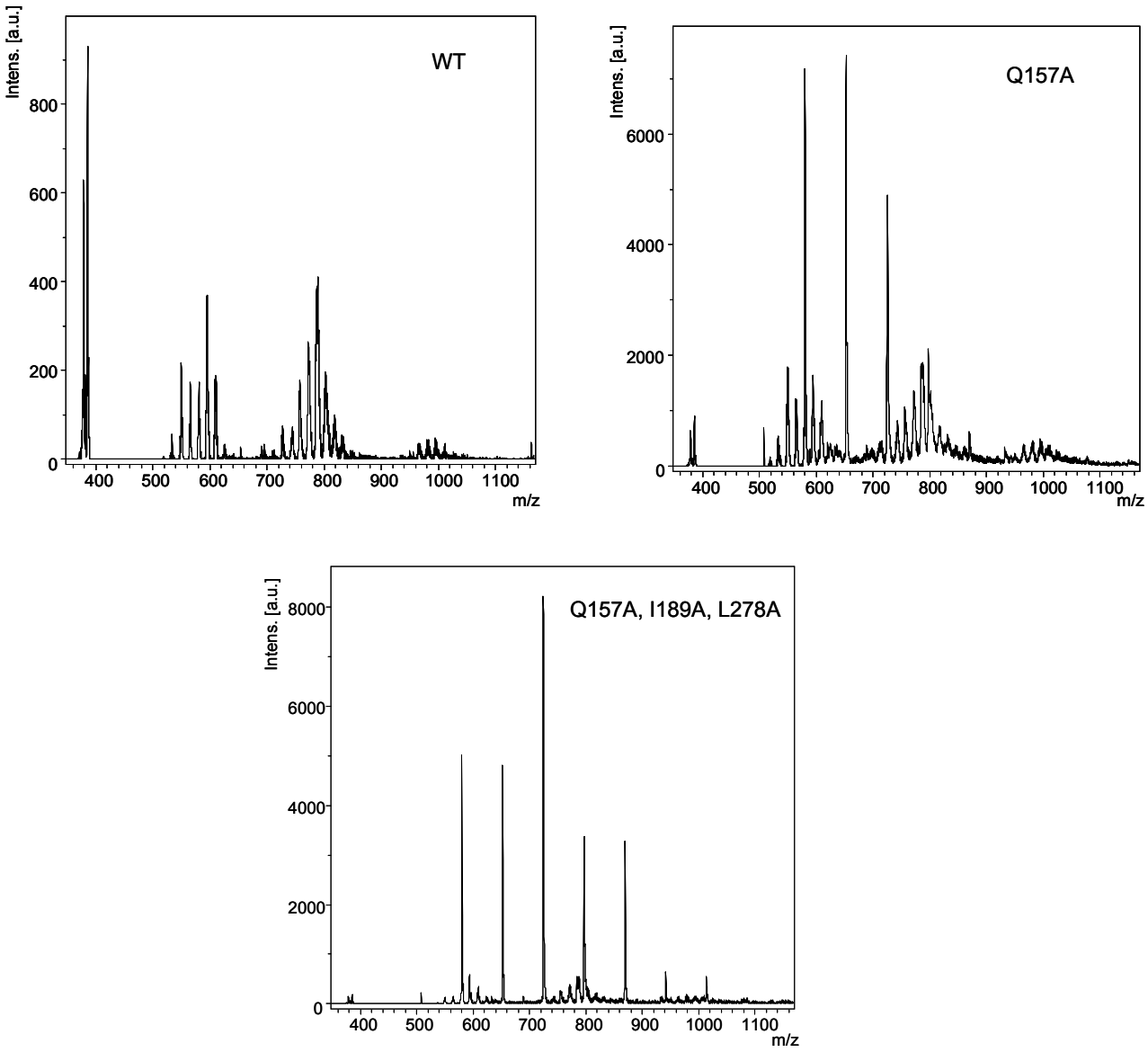

Figure 2S: MALDI-TOF-MS spectra of poly *D*,*D*-lactide synthesized by WT, mutant Q157A and mutant Q157A, I189A, L278A.

Experimental references:

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