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Rational redesign of *Candida antarctica* lipase B for the ring opening polymerization of D,D-lactide†

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Based on molecular modelling, the enzyme *Candida antarctica* lipase B was redesigned as a catalyst for the ring opening polymerization of D,D-lactide. Two mutants with 90-fold increased activity as compared to the wild-type enzyme were created. In a preparative synthesis of poly(D,D-lactide) the mutants greatly improved the rate and the degree of polymerization.

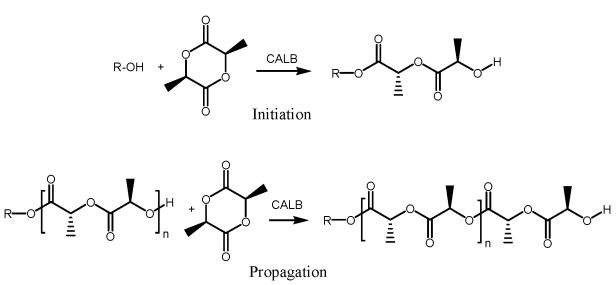
Poly(lactic acids) or poly(lactides) (PLAs) are an important subclass of the aliphatic polyester family due to their biodegradability and biocompatibility.[1,2] The production of lactic acid by fermentation of renewable agricultural sources gives them an additional benefit. PLAs find use in packaging[3] and in medical applications such as drug delivery[4] and tissue[5] and bone engineering.[6] PLAs are usually synthesised *via* polycondensation of lactic acid monomers or *via* ring opening polymerization (ROP) of lactides using metal catalysts.[7]

Enzymatic catalysis has provided new synthetic strategies for the synthesis of different types of polymers affording cleaner products under milder conditions than conventional chemical catalysis.[8] Although enzymes have been employed successfully for the synthesis of many classes of polyesters, yet no enzyme has shown any high activity for the formation of PLAs.[9] Several hydrolases, such as *Pseudomonas cepacia* lipase PS, *Humicola insolens* cutinase, *Candida cylindracea* lipase, Proteinase K, *Candida antarctica* lipase B (CALB), have been studied for the ROP of lactides.[9] Long reaction time, high background activity and high enzyme concentration have characterized these studies.[9] Recently CALB catalysed ROP resulted in no polymers when using the L,L-lactide, while 80% conversion was achieved after 2 days when using the D,D-lactide at 50 °C in toluene and 15 wt% enzyme (Novozyme 435, containing about 3 wt% active enzyme).[10]

To overcome the low enzymatic activity for the ROP of D,D-lactide lipase B from *Candida antarctica* was modified using rational redesign. The reason behind this was the successful use of CALB in the production of several types of polyesters in bulk or in solution.[11,12] Furthermore, CALB has been successfully engineered for different applications such as the change of enantioselectivity,[13] introduction of catalytic promiscuity,[14] increase of substrate specificity[15] and increase of thermal stability.[16]

Enzymatic ring opening polymerization (eROP) consists of two steps, initiation and propagation (Scheme 1). During the initiation step a nucleophile (water or alcohol) is needed for the opening of a lactide unit. In the propagation step, the hydroxyl end group of the opened lactide acts as a nucleophile for the ring opening of a new lactide unit. The ring opening of the lactide is associated with rotation of bonds, *i.e.* the C(O)–CH(Me) bond, when forming the acyl enzyme which could influence the catalysis. Jeon *et al.* have shown that CALB has a good activity for the ring opening reaction of both D,D- and L,L-lactides.[17] This indicates that the initiation step proceeds without problems and the low activity of CALB towards the ROP of lactides occurs during the propagation step (deacylation with the growing polymer as an acyl acceptor). In order to design a more active enzyme towards the D,D-lactide, a molecular modelling study was done.

The propagation step was modelled by building the substrate as a tetrahedral intermediate in the enzyme, where the free hydroxyl group of a benzyl dilactate is making a nucleophilic attack on the dilactyl enzyme (Fig. 1). The molecular dynamics simulation showed steric restrictions in the acyl donor pocket and in the entrance of the acyl acceptor pocket. These steric restrictions disturbed the formation of the hydrogen bonds essential for catalysis (shown in Fig. 1). One amino acid in the acyl donor pocket Q157 and two amino acids at the entrance of the active site I189 and L278 were suggested as targets for creating a larger space around the active site to accommodate the bulky reactants (Fig. 2). Molecular dynamics that were run



Scheme 1 The two steps of enzymatic ring opening polymerization (eROP), initiation and propagation.

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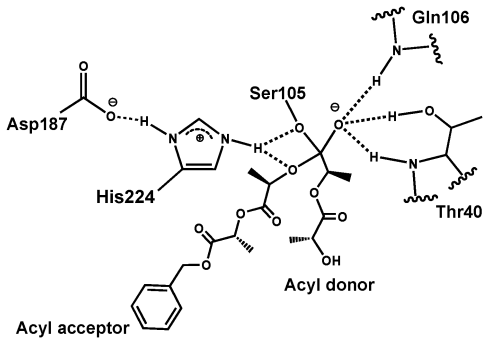

Fig. 1 Tetrahedral intermediate with benzyl D,D-dilactate (acyl acceptor) after its nucleophilic attack on the acylated enzyme. Essential hydrogen bonds for efficient catalysis are shown.

on the mutated enzyme (Q157A, I189A, L278A) showed that the substrate has gained a relaxed conformation in the active site where the acyl acceptor is pointing towards the entrance of the active site and the acyl donor had a nicer fit in the new large space in the acyl donor pocket (Fig. 2). The essential hydrogen bonds (Fig. 1) were found to be formed in the mutant enzyme during molecular dynamic simulation.

The following mutants were constructed using site directed mutagenesis: Q157A, I189A, L278A, (I189A, L278A) and (Q157A, I189A, L278A). The mutants were screened for activity towards the hydrolysis of tributyrin, the transacylation of ethyl octanoate and the ROP of D,D-lactide (Tables 1 and 2). Alanine in positions 157 and 189 caused a decrease in the hydrolysis activity towards tributyrin as compared to the wild-type enzyme (WT). The L278A mutant showed a high increase in the activity towards tributyrin in accordance with the literature.[18] The activities of the mutants for the transacylation of ethyl octanoate with 1-hexanol were all lower as compared to the WT (Table 1).

Initial screening of the mutants towards the ROP of D,D-lactide revealed that the Q157A mutant and the triple mutant (Q157A, I189A, L278A) had the highest improvements as compared to WT. These were chosen for a detailed kinetic study. The Q157A mutant showed a 90-fold improvement in the propagation step as compared to the WT (Table 2). The space created in the acyl donor pocket by replacing glutamine with alanine changed the binding orientation of the dilactyl moiety as suggested by molecular modelling (Fig. 2). This had a large impact on the propagation step by improving the deacylation rate with the growing polymer as the acyl acceptor. This was also confirmed by the mutant (Q157A, I189A, L278A) with an 83-fold rate increase as compared to the WT. In the initiation reaction the Q157A mutant showed a 4-fold increased rate as compared with WT (Table 2). The smaller increase revealed in the initiation step than in the propagation step is probably related to 1-phenylethanol as a more efficient acyl acceptor than the growing polymer. The initiation reaction was already 40-fold faster than the propagation reaction with the WT

Table 1 Rates of the hydrolysis of tributyrin and transacylation of ethyl octanoate at 25 °C catalyzed by WT CALB and mutants

	Rate[a]/s^{-1}	
Enzyme	Tributyrin[b]	Ethyl octanoate[c]
WT	330	200
Q157A	10	92
I189A	26	15
L278A	1400	16
I189A, L278A	180	5.5
Q157A, I189A, L278A	230	28

[a] (mole product) (mole catalyst)$^{-1}$ s^{-1}. [b] Hydrolysis of tributyrin determined by pH-stat. [c] Transacylation of ethyl octanoate with 1-hexanol determined by GC.

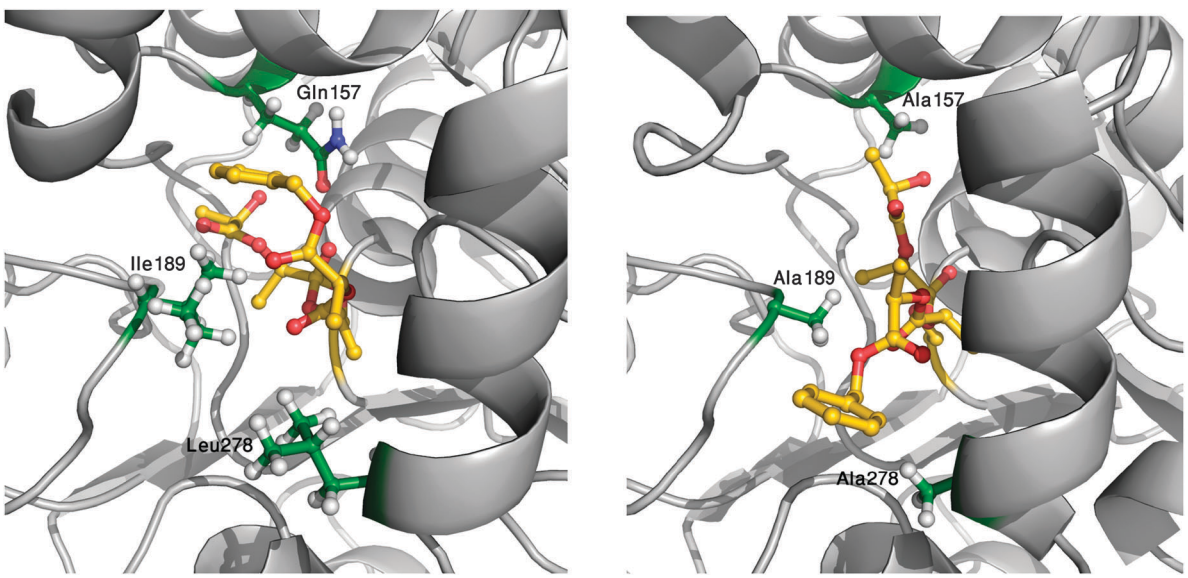

Fig. 2 Structures of the active site of the WT CALB (left) and Q157A, I189A, L278A mutant (right) with the tetrahedral intermediate representing the propagation step where a D,D-lactide unit is the acyl donor and benzyl D,D-dilactate is the acyl acceptor. The substrate in the WT (yellow, left) has a crowded conformation in the active site due to lack of space while the substrate in the mutant active site (yellow, right) has a nice fit due to the space created by mutation to alanine in positions 157, 189 and 278. The substrate is displayed without hydrogens and the side chains of the mutated amino acids are shown in green.

Table 2 Rates of the initiation and propagation steps of the ROP reaction of D,D-lactide at 60 °C catalyzed by WT CALB and mutants

Enzyme	Rate[a]/s^{-1}	
	Initiation[b]	Propagation[b]
WT	40	1
Q157A	180	93
Q157A, I189A, L278A	770	83

[a] (mole product) (mole catalyst)$^{-1}$ s^{-1}. [b] Determined by ^{1}H NMR.

Table 3 Synthesis of poly(D,D-lactide) by WT CALB and mutants[a]

Enzyme	M_n/Da		PDI[b]	DP[c]	Conv. (%)
	SEC	NMR			
WT	220	280	1.1	1	11
Q157A	560	680	1.6	4	71
Q157A, I189A, L278A	740	780	1.6	4.5	89

[a] Initiator:monomer 1:7.5. [b] Determined by SEC. [c] Determined by ^{1}H NMR.

enzyme. The triple mutant (Q157A, I189A, L278A) showed another 4-fold increase in the initiation step as related to the Q157A mutant (Table 2). The additional space created around the entrance of the active site having alanine in positions 189 and 278 made the initiation step more efficient. In contrast to the increased ROP activity of the lactide, the activity towards ethyl octanoate was found to be 50% for the Q157A mutant and 15% for the triple mutant (Q157A, I189A, L278A) as compared to WT (Table 1). The created space in the enzyme had thus a negative influence on the enzyme activity towards ethyl octanoate, a substrate with already a good fit in the active site of the WT enzyme.

Preparative ROP reactions were run using the WT and mutants Q157A and (Q157A, I189A, L278A). The reactions were allowed to run for 48 hours in D_8-toluene at 60 °C using 1-phenylethanol as an initiator. The synthesized polymers were characterized by NMR, MALDI-TOF and SEC (ESI†). As shown in Table 3, the highest conversion (89%) was achieved with mutant (Q157A, I189A, L278A) and polymers with molecular weight (M_n) of 780 Da were produced corresponding to a degree of polymerization (DP) of 4.5 lactide units (9 lactic acid units) and PDI of 1.6. Mutant Q157A gave a conversion of 70% and polymers with M_n of 680 Da, DP of 4 and PDI of 1.6 (Table 3). The WT showed 11% conversion and polymers with M_n of 280 Da, DP of 1 which indicate that the WT has catalyzed only the initiation step. The DP was lower than expected probably due to water initiation. MALDI-TOF analysis of the polymers produced by the mutants Q157A and (Q157A, I189A, L278A) showed a main group of peaks (ESI†) with a mass difference of 72 Da, which corresponds to one lactic acid unit. This shows that the mutants used the polymer product for transacylation during the polymerization reaction in line with other eROP results.[19] No peaks corresponding to poly(lactide) were detected in the WT reaction.

In conclusion, CALB was rationally redesigned and two variants Q157A and (Q157A, I189A, L278A) showed 90-fold improvement in the ROP activity towards D,D-lactide as compared to the WT enzyme. Molecular dynamics simulation suggested that the larger space created by the Q157A mutation altered the orientation of the dilactyl moiety in the tetrahedral intermediate. This improved the deacylation step of the propagation in the ROP reaction drastically. On the other hand, for ethyl octanoate (a good substrate for the WT enzyme) the large space caused in the mutants resulted in decreased transacylation activity towards 1-hexanol as compared to the WT enzyme.

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Notes and references

1 J. R. Dorgan, H. J. Lehermeier, L.-I. Palade and J. Cicero, *Macromol. Symp.*, 2001, **175**, 55.
2 M. Vert, S. M. Li, G. Spenlehauer and P. Guerin, *J. Mater. Sci.: Mater. Med.*, 1992, **3**, 432.
3 R. Auras, B. Harte and S. Selke, *Macromol. Biosci.*, 2004, **4**, 835.
4 S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang and R. Langer, *Pharm. Res.*, 1991, **8**, 713.
5 W. Murphy, M. Peters, D. Kohn and D. Mooney, *Biomaterials*, 2000, **21**, 2521.
6 A. Majola, S. Vainionpää, K. Vihtonen, M. Mero, J. Vasenius, P. Törmälä and P. Rokkanen, *Clin. Orthop. Relat. Res.*, 1991, **268**, 260.
7 O. Dechy-Cabaret, B. Martin-Vaca and D. Bourisso, *Chem. Rev.*, 2004, **104**, 6147.
8 S. Kobayashi and A. Makino, *Chem. Rev.*, 2009, **109**, 5288.
9 S. Matsumura, K. Mabuchi and K. Toshima, *Macromol. Rapid Commun.*, 1997, **18**, 477; S. Matsumura, K. Tsukada and K. Toshima, *Int. J. Biol. Macromol.*, 1999, **25**, 161; K. Numata, R. K. Srivastava, A. Finne-Wistrand, A. C. Albertsson, Y. Doi and H. Abe, *Biomacromolecules*, 2007, **8**, 3115; S. Huijser, B. B. P. Staal, J. Huang, R. Duchateau and C. E. Koning, *Biomacromolecules*, 2006, **7**, 2465; R. Garcia-Arrazola, D. A. López-Guerrero, M. Gimeno and E. Bárzana, *J. Supercrit. Fluids*, 2009, **51**, 197.
10 M. Hans, H. Keul and M. Moeller, *Macromol. Biosci.*, 2009, **9**, 239.
11 S. Kobayashi, *Macromol. Rapid Commun.*, 2009, **30**, 237.
12 M. Takwa, K. Hult and M. Martinelle, *Macromolecules*, 2008, **41**, 5230.
13 A. Magnusson, M. Takwa, A. Hamberg and K. Hult, *Angew. Chem., Int. Ed.*, 2005, **44**, 4582.
14 M. Svedendahl, B. Jovanovic, L. Fransson and P. Berglund, *ChemCatChem*, 2009, **1**, 252.
15 P.-O. Syrén, E. Lindgren, H. Wolfgang Hoeffken, C. Branneby, S. Maurer, B. Hauer and K. Hult, *J. Mol. Catal. B: Enzym.*, 2010, **65**, 1.
16 H. S. Kim, Q. A. T. Le and Y. H. Kim, *Enzyme Microb. Technol.*, 2010, **47**, 1.
17 N. Y. Jeon, S.-J. Ko, K. Won, H.-Y. Kang, B. T. Kim, Y. S. Leea and H. Lee, *Tetrahedron Lett.*, 2006, **47**, 6517.
18 D. Liu, P. Trodler, S. Eiben, K. Koschorreck, M. Müller, J. Pleiss, S. C. Maurer, C. Branneby, R. D. Schmid and B. Hauer, *ChemBioChem*, 2010, **11**, 789.
19 M. Takwa, Y. Xiao, N. Simpson, E. Malmström, K. Hult, C. E. Koning, A. Heise and M. Martinelle, *Biomacromolecules*, 2008, **9**, 704.

