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Increased activity of enzymatic transacylation of acrylates through rational design of lipases

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ABSTRACT

A rational design approach was used to create the mutant *Candida antarctica* lipase B (CALB, also known as *Pseudozyma antarctica* lipase B) V190A having a k_{cat} three times higher compared to that of the wild type (wt) enzyme for the transacylation of the industrially important compound methyl methacrylate. The enzymatic contribution to the transacylation of various acrylates and corresponding saturated esters was evaluated by comparing the reaction catalysed by CALB wt with the acid (H_2SO_4) catalysed reaction. The performances of CALB wt and mutants were compared to two other hydrolases, *Humicola insolens* cutinase and *Rhizomucor mihei* lipase. The low reaction rates of enzyme catalysed transacylation of acrylates were found to be caused mainly by electronic effects due to the double bond present in this class of molecules. The reduction in rate of enzyme catalysed transacylation of acrylates compared to that of the saturated ester methyl propionate was however less than what could be predicted from the energetic cost of breaking the π-system of acrylates solely. The nature and concentration of the acyl acceptor was found to have a profound effect on the reaction rate.

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1. Introduction

Acrylates are industrially important compounds that have a wide range of commercial applications. The α,β-double bond present in these molecules can be used for radical polymerization to yield final products such as paints, plastics and adhesives to be used for example in the automotive industry. Traditionally, esters of acrylic acid and methacrylic acid are made chemically at elevated temperature, with polymerization inhibitors and with sulphuric acid as catalyst [1,2]. Biocatalysis is a "green" option to traditional chemistry and could provide sustainable routes to acrylic esters starting from renewable sources such as glucose and lactate [3,4]. Enzymes are attractive since they allow the use of mild reaction conditions, often displaying high activity as well as high regio-/stereo selectivity [5,6]. Especially lipases are of industrial interest due to their high stability and activity in organic media.

Lipase catalysed transacylation of acrylates has been described in the literature. In a pioneering work regioselective acrylation of sterically hindered diols was performed using a lipase from *Chromobacterium viscosum* [7]. Lipase B from *Candida antarctica* (CALB, also known as *Pseudozyma antarctica* lipase B, EC 3.1.1.3) was shown to be a superior catalyst out of 19 enzymes investigated in the enzyme catalysed transacylation of methyl acrylate with 1-undecanol [8]. The industrial interest in enzymatic acrylation is shown in the number of patents on the subject. Enzyme catalysed regioselective transacylation of acrylates with glucosides, with the aim of producing polymeric sugar acrylates, as well as monoacrylation of polyols has been described by BASF [9,10]. One limitation of enzyme catalysed acrylation is the low reaction rates compared to the rates for corresponding saturated esters. Attempts have been made to increase the reaction rate of enzymatic acrylation, mainly by the choice of solvent and by optimisation of the water activity [11,12]. The source of the low rates has to our knowledge not been discussed.

In this work we have employed a rational design approach based on computer modelling with the aim of increasing the enzymatic activity (k_{cat}) for methyl methacrylate. The analysis of the CALB mutants D134N, I189A, I189V, V190A, V190L, and I285W gave valuable insight into the problems associated with enzymatic acrylation. *Humicola insolens* cutinase (EC 3.1.1.74) and *Rhizomucor miehei* lipase (EC 3.1.1.3) were compared to CALB regarding

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performance in enzymatic acrylation. The enzymatic contribution to the transacylation of methyl acrylate and derivatives as well as to corresponding saturated esters was evaluated.

Alcohols are known to inhibit lipases by forming a dead-end complex with the enzyme [13]. The protein engineering approach to enhance enzymatic activity was compared to a reaction engineering approach. The nature and concentration of the alcohol acyl acceptor was found to have a profound effect on the reaction rate for enzyme catalysed acrylation.

2. Experimental

2.1. Chemicals and enzymes

Methyl propionate, methyl isobutyrate, methyl trimethylacetate, methyl acrylate, methyl crotonate, methyl methacrylate, 1-propanol, 2-butanol, acetonitrile (for fluorescence) and dodecane of ≥99% purity and $H_2SO_4 \geq 95\%$ purity were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl α-chloro acrylate (≥99%), stabilised with hydroquinone, was from Acros Organics (Morris Plains, NJ, USA). Diisopropyl ether (≥99%) was from AppliChem (Darmstadt, Germany). 2-Ethyl-1-hexanol was from BASF (Ludwigshafen, Germany).

H. insolens cutinase wt was a kind gift from Novozymes (Bagsvaerd, Denmark). Immobilised *R. miehei* lipase was purchased from Sigma–Aldrich. Restriction enzymes were from Fermentas (St. Leon-Rot, Germany).

2.2. Preparation of C. antarctica lipase B wt and mutants

The preparation of the CALB wt (also known as *Pseudozyma antarctica* lipase B) construct in a pGAPzαβ plasmid and its transformation into the expression host *Pichia pastoris* has been described previously in detail [14]. The QuikChange® protocol from Stratagene (La Jolla, CA, USA) was used to introduce mutations. The mutagenic primers where designed to be non-overlapping in the ends [15] and were ordered from Thermo Fisher Scientific (Ulm, Germany). The DNA-sequences in the 5′–3′ direction for the forward (f) and reverse (r) primers are given below. Introduced alterations in the wt sequence are in uppercase letters for clarity.

D134N_f
5′-ggcctttgcgcccAactacaagggcaccgtcctcg-3′
D134N_r
5′-ggtgcccttgtagtTgggcgcaaaggccataagtcgatcg-3′
I189V_f
5′-cgaccgacgagGtcgttcagcctcaggtgtcc-3′
I189V_r
5′-cctgaggctgaacgaCctcgtcggtcgccg-3′
I189A_f
5′-cggcgaccgacgagGCcgttcagcctcaggtgtcc-3′
I189A_r
5′-cctgaggctgaacgGCctcgtcggtcgccgagtagagg -3′
V190A_f
5′-cgacgagatcgCtcagcctcaggtgtcc-3′
V190A_r
5′-cctgaggctgaGcgatctcgtcggtcg-3′
V190L_f
5′-cgaccgacgagatcCttcagcctcaggtgtccaactcg-3′
V190L_r
5′-cctgaggctgaaGgatctcgtcggtcgccgagtagagg-3′
I285W_f
5′-gctgcagccTGGgtggcgggtccaaagcagaactgc-3′
I285W_r
5′-ggacccgccacCCAggctgcagctgccggcgccaggagc-3′

The PCR reactions were analysed on a 1% agarose gel for the presence of products. After DpnI digestion, the mutated plasmid DNA as well as the wt plasmid DNA was transformed by heat-shock into *E. coli* XL1Blue cells according to the QuikChange protocol. All mutations were confirmed by sequencing. Prior to expression, the wt and mutated pGAPzαβ plasmids were harvested by the QIAprep Miniprep System (QIAGEN GmbH, Hilden, Germany). Around 20–40 μg plasmid DNA was linearized by Bsp HI overnight at 37 °C and concentrated by EtOH precipitation according to standard protocols. Approximately 10 μg of the linearized plasmid was electroporated into freshly made *Pichia pastoris* SMD1168H competent cells using the following settings: 1.5 kV, 400 Ω, 25 μF. Ice-cold 1 M sorbitol was immediately added after transformation. After 2 h at 30 °C, half of the transformation mixture was plated on YPDS-plates (1% yeast extract, 2% peptone, 2% dextrose (D-glucose), 1 M sorbitol, 2% agar) containing 100 μg/mL zeocin. The other half of the mixture was diluted with YPD-media (1% yeast extract, 2% peptone, 2% dextrose (D-glucose)) and was allowed to shake at 200 rpm for 3 h at 30 °C after which plating was performed (on YPDS-plates containing 100 μg/mL zeocin). Around 10 colonies were selected and grown in 10 mL YPD for approximately 36 h. After centrifugation at 4000 rpm, 4 °C, for 10 min using a Sorvall Super T21 centrifuge, the culture supernatants were analysed by SDS-gel electrophoresis (NuPAGE® 10% Bis-Tris gel, Invitrogen, Carlsbad, CA, USA) using MOPS as running buffer (MOPS 50 mM; Tris base 50 mM; SDS 0.1%; EDTA 1 mM; pH 7.7). The validity of the *C. antarctica* lipase B protein band was established by performing Western blot analysis using antibodies against CALB as previously described [14].

2.3. Protein expression and purification

Positive *Pichia pastoris* clones expressing CALB variants were grown on YPD-agar plates containing 100 μg/mL zeocin. After 2 days, colonies were picked and grown for 24 h in 5 mL YPD at 30 °C, 200 rpm. A fraction of the pre-cultures (to obtain a 1:100 dilution) were transferred to 50–500 mL BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base with ammonium sulphate without amino acids, 0.4 mg/L (4×10^{-5}%) biotin, 1% glycerol, pH 6.0). The main cultures were allowed to grow for 3–4 days at 30° at 200–260 rpm after which the optical density (OD_{600}) typically reached ≥40. The cultures were centrifuged at 4000 rpm, 4 °C for 20 min. Protein purification was performed using hydrophobic interaction chromatography (HIC) as previously described [16] with minor modifications. Ammonium acetate (9 M) was slowly added under stirring to the supernatant to a final concentration of 0.8 M. The feed-stock was filtered through a 0.2 μM bottle-top filter (Sartorius AG, Goettingen, Germany) and loaded onto an XK-16 column packed with 15 mL butyl sepharose FF and connected to an ÄKTA explorer (GE Healthcare, Uppsala, Sweden). The equilibration buffer was 50 mM potassium phosphate, 0.8 M ammonium acetate, pH 6. A linear gradient to 50 mM potassium phosphate, pH 6 was used for elution and finally milliQ was used to elute remaining protein from the column. Fractions giving raise to an A_{280}-signal were pooled and concentrated (by a factor of 10) with 50 mM potassium phosphate, pH 7.5 by using Centricon Centrifugal filters with an MWCO of 5 kDa (Millipore, MA, USA). The purity of the CALB variants was assessed by SDS polyacrylamide gel electrophoresis. Purification typically resulted in 10–20 mg pure protein/L media.

2.4. Protein immobilization and active site titration

Lipase immobilization and active site titration have previously been described by our group [17]. *C. antarctica* lipase B wt and mutants as well as *H. insolens* cutinase were immobilized on Accurel MP 1000 < 1500 μm (Accurel Systems, Sunnyvale, CA, USA). The supernatant was confirmed to be devoid of lipase activity after immobilization. The lipase-containing polypropylene beads were vacuum-dried overnight and put in a desiccator under LiCl(s) to ensure a water activity of 0.1.

The suicide inhibitor 4-methylumbelliferyl hexylphosphonate was added to 10–50 mg polypropylene beads containing lipase in acetonitrile. The final concentration of the inhibitor in the experiments was 50 μM and the total volume was 1 mL. The fluorescence intensity was analysed by adding 100 μL sample solution to 900 μL buffer containing 100 mM Tris–HCl, 1 mM $CaCl_2$, pH 8.0 and by measuring the signal on a luminescence spectrometer (Luminescence Spectrometer Model LS-50B, PerkinElmer, MA, USA). Excitation was at 360 nm and the emission wavelength was 445 nm. The amount of active sites were calculated from a standard curve of the linear relationship between fluorescense intensity and the concentration of the leaving group 4-methylumbelliferone. Resulting protein load on the support for *C. antarctica* lipase B was found to be (% weight/weight): 0.5% for wt, 0.5% for D134N, 0.3% for I189A, 0.6% for I189 V, 0.2% for V190A, 1% for V190L and 0.8% for I285W. The corresponding numbers were 0.5% for *H. insolens* cutinase and 0.2% for *R. miehei* lipase.

2.5. Kinetic measurements

All reactions were performed in glass tubes with screw-lids. All glassware was dried at 250° prior to use and chemicals were dried by molecular sieves (3 A, Sigma–Aldrich).

The relative substrate specificities were measured by competition experiments where several acyl donors were used simultaneously in the same reaction tube. The temperature was set to 30 °C using a water bath and magnetic stirring was used. Typically, the acyl donor concentration was in the range of 50–200 mM and the acyl acceptor 1-propanol had a concentration of 100 mM. Dodecane was used as internal standard at a concentration of 2.5 mM. Diisopropyl ether was chosen as solvent, being moderately hydrophobic and able to dissolve all substrates and products. The total reaction volume was 2 mL. The relative specificities were not dependent on the alcohol concentration used. As catalysts, 8% vol./vol. H_2SO_4 (resulting in relative second-order rate constants) or 10 mg beads with immobilized lipase (CALB wt) were used (resulting in relative k_{cat}/K_M values). Samples from the reaction mixtures were taken at regular time intervals and diluted 10 times in diisopropyl ether. The amount of product (propyl esters) was determined by GC-analysis using a HP 5890 Series II Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA). GC-columns were Varian Chrompack Capillary column, WCOT fused silica, 25 m × 0.32 mm in diameter, CP Chirasil-DEX CB, DF = 0.25 and Chrompack WCOT fused silica 25 m × 0.32 mm in diameter, CP-SIL 5CB, DF = 1.2 respectively. The initial rate was calculated from data of product concentration as a function of time. Only data corresponding to a conversion of 10% or less were considered. The relative k_{cat}/K_M values were calculated from Eq. (1) where A and B are the two acyl donors being compared and ν_A and ν_B the initial rates for the production of the corresponding propyl esters. The same equation is valid for the acid catalysed reaction if the second-order rate constants are used.

$$\frac{(k_{cat}/k_M)_A}{(k_{cat}/k_M)_B} = \frac{\nu_A}{\nu_B} \times \frac{[B]}{[A]} \quad (1)$$

Absolute k_{cat}/K_M values for methyl propionate were determined for the various lipases using 25 mM 1-propanol as acyl acceptor at 30 °C. The concentrations of active sites determined by active site titration were taken into account. The known relative k_{cat}/K_M values were then used to obtain the absolute k_{cat}/K_M values at 25 mM 1-propanol for the other substrates.

The apparent k_{cat} values and rate constants for enzymatic transacylation of methyl propionate, methyl acrylate, methyl methacrylate and methyl α-chloro acrylate were measured at 30° in diisopropyl ether as solvent with 25 mM 1-propanol as nucleophile and 2.5 mM dodecane as internal standard. Optionally, 2-butanol was used as acyl acceptor at a concentration of 470 mM. Typically, the acyl donor concentration was varied from 50 mM to bulk-conditions (when the acyl donor is the solvent). The apparent k_{cat} values were calculated by fitting the experimental data to pseudo one-substrate Michaelis–Menten kinetics using non-linear regression in the solver function of Microsoft Excel 2003. At least 6 points were used to create a Michaelis–Menten curve. The rate constants were measured using the acyl donor as solvent (from at least 2 separate reactions). Turnover of substrate never exceeded 10% conversion and the concentrations of active sites were taken into consideration when calculating apparent rate constants. The temperature was kept constant at 30 °C and the shaking speed was 100 rpm using an HLC heat-block. Typically 10 mg Accurel MP 1000 with immobilised enzyme (approximately 1 nmol active site) was used for each reaction mixture.

Apparent K_M values were either determined from Michaelis–Menten curves or estimated from known absolute k_{cat}/K_M values and known rates in bulk (which was then set to represent k_{cat}).

The relative initial rate of CALB catalysed transacylation of methyl methacrylate as a function of the mole fraction of 1-propanol was performed in a solvent free system at 30 °C. Corresponding experiments were performed using 2-butanol and 2-ethyl-1-hexanol as acyl acceptors.

Background reactions were in all cases found to be negligible.

2.6. Computer modelling on C. antarctica lipase B

The structure of the tetrahedral intermediate as a model of the transition state (TS) for acylation of methyl methacrylate catalysed by *C. antarctica* lipase B was prepared using YASARA version 8.9.23 [18]. The PDB-file 1TCA was used as starting structure. Sugars were deleted, all missing hydrogens were added and the hydrogen bonding network was optimised by repeated minimizations and short dynamics runs (1200 fs at 298 K) using the Amber99 force field [19] and by first keeping the protein backbone fixed. Free methyl methacrylate was built and energy minimised in YASARA using the AUTOSMILES approach [20]. The tetrahedral intermediate was then created by the covalent attachment of the carbonyl carbon in methyl methacrylate to the catalytic S105 in CALB. The resulting tetrahedral intermediate was minimized and molecular dynamics was run on the covalent enzyme–substrate complex for 2 ps at 298 K using Amber99 and PME for long range electrostatics [21]. This was repeated several times and the molecular dynamics simulation was allowed to proceed until (1) the oxyanion hole (Q106 and T40) was clearly established and contributing with at least two hydrogen bonds to the (former) carbonyl oxygen and (2) the hydrogen bonds in the catalytic triad formed (D187 hydrogen bonded to H224 and H224 sharing a hydrogen bond between the ester oxygen in methyl methacrylate and the O_γ of the catalytic serine). The catalytic histidine was in its protonated state. To explore the conformational space the dihedral angle between the carbonyl oxygen, carbonyl carbon, and the unsaturated α- and β-carbons in the acrylate substrate in TS was manually varied followed by minimization and molecular dynamics without constraints at 298 K (for 2 ps). The aim of the simulations was to get a visual picture of potential problematic interactions in TS.

3. Results and discussion

3.1. Comparison of acid and enzyme catalysed transacylation of acrylates and corresponding saturated esters

To evaluate the contribution of lipases in transacylation reactions of acrylates in relation to methyl propionate the enzyme

catalysed reaction was compared to the reaction catalysed by H_2SO_4. The apparent second-order rate constant for the enzyme (k_{cat}/K_M) was compared to the corresponding second-order rate constant for the acid. The relative second-order rate constants for H_2SO_4-catalysed transacylation of methyl acrylate, methyl methacrylate (relative second-order rate constant $k = 1$), methyl crotonate, methyl α-chloro acrylate as well as for the corresponding saturated esters are given in Table 1 together with the corresponding relative k_{cat}/K_M values for *C. antarctica* lipase B (with the value for methyl methacrylate also set to 1).

Methyl propionate was chosen as reference substrate for the lipase catalysed transacylation reactions since it is the corresponding saturated ester of methyl acrylate and is more similar to natural substrates. The reaction rate for acid catalysed transacylation of methyl methacrylate was 5–6 times lower than for methyl acrylate. The corresponding figure for CALB was a 17-fold reduction. This finding is encouraging since it indicates that the enzymatic transacylation of methyl methacrylate could in theory be improved at least three times if the normal capacity of the enzyme could be used. We believe that sterical hindrance around the α-carbon of the substrate as well as electronic effects are important. Acrylates have been shown to predominantly exist in two flat conformations, s-*cis* and s-*trans* (which indicates the relative orientation of the two double bonds) [22]. Substrate geometrical effects can be seen in the fact that the enzyme specificity for methyl isobutyrate was a magnitude larger than for the iso-steric compound methyl methacrylate. In the acid catalysed reaction methyl trimethylacetate displayed a twofold higher reactivity than methyl methacrylate whereas the enzyme had very low specificity towards this bulky substrate. Interestingly, the enzyme specificity for methyl crotonate was higher than for methyl methacrylate. This was also true for methyl α-chloro acrylate, indicative of an electronic effect since the size of the chlorine is close to that of the methyl group. The relative enzymatic contribution is graphically visualized in Fig. 1. It can be concluded that CALB is not working at its maximal capacity for substrates with sterical hindrance at the α-position when taking the intrinsic chemical reactivities of the substrates into account. It should also be noted that the relative enzymatic contribution to catalysis for methyl propionate and methyl acrylate were essentially the same.

3.2. Molecular model of the transition state of CALB-catalysed acylation of methyl methacrylate

The tetrahedral intermediate has been shown to be a good approximation of the transition state (TS) for lipase catalysed acylation [23]. Fig. 2 shows a graphical representation of the TS for

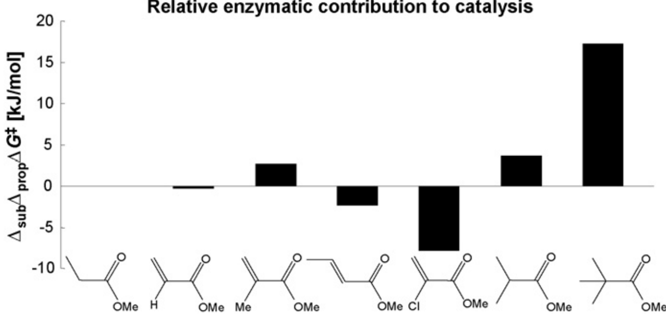


Fig. 1. Relative enzymatic contributions were calculated from the apparent second-order rate constants for enzyme (CALB wt) and acid (H_2SO_4) catalysed transacylation (k_{cat}/K_M *and* k respectively). The enzymatic contribution to the transacylation of methyl propionate was used as reference (relative contribution set to 0). Negative relative energies are associated with higher degree of enzymatic contribution $\Delta_{sub} = \Delta^{substrate}_{Acid\text{-}Enzyme}$; $\Delta_{prop} = \Delta^{Methyl\ propionate}_{Acid\text{-}Enzyme}$.

Table 1
Relative specificity for acid and lipase catalysed transacylation of acrylates and corresponding saturated esters in diisopropyl ether using 25 mM 1-propanol as acyl acceptor at 30 °C.

Catalyst	OMe	H, OMe	Me, OMe	OMe	Cl, OMe	OMe	OMe
H_2SO_4, k [a]	54	5.6	1	2.4	0.5	12	2
CALB wt,[b] k_{cat}/K_M	150	17	1	21	41	12	0.004
Relative to methyl propionate[c]							
H_2SO_4, k	100	10	1.9	4.4	0.9	22	3.7
CALB wt, k_{cat}/K_M	100	11	0.7	14	27	8	0.003

[a] Relative second-order rate constants with $k = 1$ for methyl methacrylate using 8% vol./vol. H_2SO_4.
[b] Relative k_{cat}/K_M values for *Candida antarctica* lipase B with k_{cat}/K_M set to 1 for methyl methacrylate.
[c] The relative second-order rate constants (for H_2SO_4) and relative k_{cat}/K_M values (for *Candida antarctica* lipase B) with the values for methyl propionate set to 100%.

Table 2
Absolute apparent k_{cat}/K_M values for enzyme catalysed transacylation in diisopropyl ether using 25 mM 1-propanol at 30 °C.

Catalyst	OMe	H, OMe	Me, OMe	OMe	Cl, OMe	OMe	OMe
	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]	k_{cat}/K_M [M^{-1} min^{-1}]
CALB wt[a]	5500	630	37	790	1500	450	0.1
I189A[a]	800	80	6	120	200	90	0.7
I189V[a]	4300	390	20	470	740	330	0.1
V190A[a]	1500	160	18	180	840	190	0.2
Cutinase[b]	10000	1200	145	1200	6200	4000	n.m.[d]
RML[c]	9500	100	44	40	850	2200	n.m.[d]

[a] *Candida antarctica* lipase B.
[b] *Humicola insolens* cutinase.
[c] *Rhizomucor miehei* lipase.
[d] Not measured.

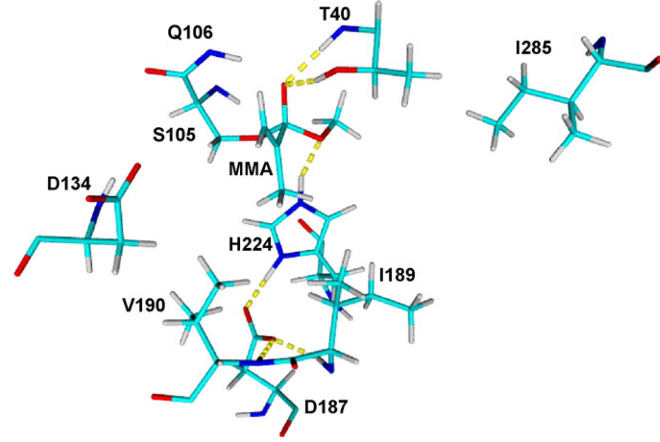


Fig. 2. A minimised snapshot of the tetrahedral intermediate from the dynamics simulation of covalently attached methyl methacrylate (MMA) in CALB. Residues targeted for mutation are shown (D134, I189, V190, I285). The catalytic triad (S105, D187, H224) and oxyanion hole (T40, Q106) are shown. Atoms are coloured according to type.

CALB wt-catalysed acylation of methyl methacrylate (one energy minimised snapshot from the molecular dynamics simulation).

The α-methyl group in methyl methacrylate is in proximity of I189 and V190 (closest distance between carbon atoms are 3.6 Å in both cases). From this simple model it can be concluded that the mutations I189 V, I189A, V190A and V190L could relieve potential sterical clashes in TS. D134N changes the electronic environment around the substrate and I285W could potentially introduce π-stacking interactions.

3.3. Performances of lipases in enzymatic acrylation

The performances of CALB wt and mutants, *H. insolens* cutinase and *R. miehei* lipase were analysed in transacylation reactions of acrylates and corresponding saturated compounds. Substrate specificities are given in Table 2. The first observation is that the specificities were low and very far from the diffusion limit of 10^{10} M^{-1} min^{-1} [24]. The specificities dropped one order of magnitude for methyl acrylate compared to methyl propionate and two orders of magnitude for methyl methacrylate compared to methyl propionate for all enzymes tested. The enzyme specificity for methyl isobutyrate was one order of magnitude larger than for the isosteric methyl methacrylate. We believe that the flat s-*cis*/s-*trans* acrylate conformers interact unfavourably with the active site of lipases. Methyl isobutyrate is not forced to be in a planar arrangement. *H. insolens* cutinase seemed to have a higher intrinsic specificity for acrylates compared to the other enzymes investigated. *R. miehei* lipase displayed a k_{cat}/K_M ratio of methyl acrylate and methyl methacrylate of two, which is lower than the relative chemical reactivity of these substrates. Clearly, three methyl groups on the α-position as in methyl trimethylacetate was too bulky for CALB.

Enzyme substrate specificities (k_{cat}/K_M) tell something about both binding and catalysis and corresponds to the observed rate constant at very low substrate concentrations. For industrial processes, maximal rates are of high interest since they indicate how many molecules that can be converted into product per unit time and per enzyme molecule under saturating conditions. By using bulk-conditions (i.e. using the acyl donor as solvent) such maximal rates can be estimated (in cases where substrate inhibition is not predominant). Rate constants for enzymatic transacylation of acrylates and for methyl propionate are given in Table 3. The reactions were performed in bulk with 25 mM 1-propanol as acyl acceptor. The rate constant for methyl acrylate was observed to be

Table 3
Rate of enzymatic transacylation in pure acyl donor using 25 mM 1-propanol as acyl acceptor at 30 °C.

Catalyst	O, OMe rate [min^{-1}]	O, H, OMe rate [min^{-1}]	O, Me, OMe rate [min^{-1}]	O, Cl, OMe rate [min^{-1}]
CALB wt[a]	1600	580	60	290
I189A[a]	300	170	30	n.m.[b]
I189V[a]	800	240	25	n.m.[b]
V190A[a]	1300	240	85	600
Cutinase[c]	1000	170	80	280
RML[d]	3000	35	50	120

[a] *Candida antarctica* lipase B.
[b] Not measured.
[c] *Humicola insolens* cutinase.
[d] *Rhizomucor miehei* lipase.

Table 4
Apparent K_M values using 25 mM 1-propanol and diisopropyl ether as solvent at 30 °C.

Catalyst	O, OMe	O, H, OMe	O, Me, OMe	O, Cl, OMe
	K_M^{app} [M]	K_M^{app} [M]	K_M^{app} [M]	K_M^{app} [M]
CALB wt[a]	0.2[b]	0.6[b]	1.5[b]	0.1[b]
I189A[a]	0.4	2	>13	n.m.[c]
I189V[a]	0.2	0.6	1.4	n.m.[c]
V190A[a]	0.9	1.5	10[b]	1[b]
Cutinase[d]	0.1	0.2	0.6	0.05
RML[e]	0.3	0.4	1.3	0.1

The apparent K_M values were estimated from known absolute k_{cat}/K_M values and the rate constants from reactions where the acyl donor was used as solvent or from Michaelis-Menten data as indicated.
[a] *Candida antarctica* lipase B.
[b] K_M values from Michaelis–Menten kinetics using 25 mM 1-propanol and diisopropyl ether as solvent at 30 °C.
[c] Not measured.
[d] *Humicola insolens* cutinase.
[e] *Rhizomucor miehei* lipase.

around 6 times lower than for methyl propionate (with the exception of *R. miehei* lipase where the decrease in rate was 85 times). The stabilising π-system present in methyl acrylate is worth around 17 kJ/mol in diethyl ether [25]. The conjugated π-system has to be broken during the course of the reaction so the observed rate for transacylation of methyl acrylate is higher than expected (17 kJ/mol would correspond to a 1000-fold reduction in rate). The conversion of methyl methacrylate to propyl methacrylate proceeded slower by an order of magnitude compared to methyl acrylate, indicative of steric hindrance. Interestingly, *R. miehei* lipase displayed a slightly higher rate for the transacylation of methyl methacrylate as compared to methyl acrylate. Methyl α-chloro acrylate displayed a higher rate compared to methyl methacrylate indicative of an electronic effect.

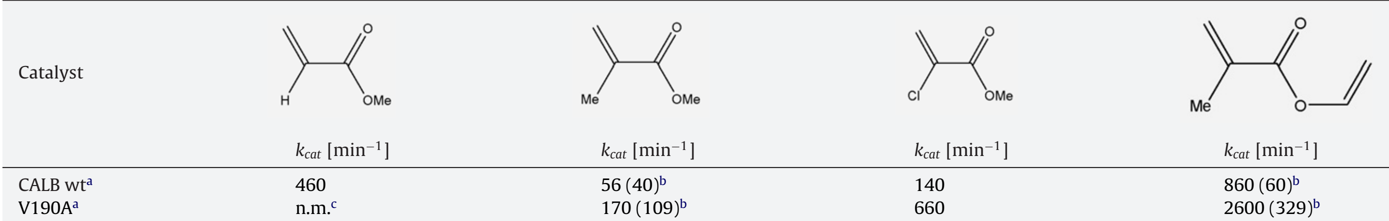
The apparent estimated K_M values for the acrylates and the reference compound methyl propionate are given in Table 4. It is seen that poor substrate binding is a problem in enzymatic acrylation. This is clearly illustrated for methyl methacrylate with a K_M of 1.5 M for CALB wt and 10 M for the CALB V190A mutant (pure methyl methacrylate has a concentration around 9 M). Clearly, the mutations I189A and V190A were deleterious for substrate binding.

Apparent k_{cat} values for CALB-catalysed transacylation of acrylates are given in Table 5 with the values using 2-butanol as acyl acceptor given within brackets. CALB V190A showed three times higher reactivity towards methyl methacrylate compared to wt. By using the activated ester vinyl methacrylate and 1-propanol, the apparent k_{cat} value increased 10-fold compared to methyl methacrylate for both CALB wt and the V190A mutant. It should be noted that the apparent K_M value for vinyl methacrylate was around 10 M for both wt and mutant using 1-propanol. The observed rate increase was thus a “real” effect and not caused by successful competition of the activated ester with the alcohol inhibitor. The acylation step of enzymatic acrylation was thus concluded to be more rate-limiting using 1-propanol as acyl acceptor. When using 1-hexanol as acyl acceptor the rate of *C. antarctica* lipase B catalysed transacylation of methyl methacrylate was essentially unchanged. For 2-butanol, the effect of switching to the activated ester vinyl methacrylate had a less profound effect with only moderate increase in reaction rate compared to methyl methacrylate. This is indicative that deacylation has become more important and consistent with the fact that 2-butanol is a less potent nucleophile compared to 1-propanol. Interestingly by using 2-butanol as a nucleophile, the apparent K_M value for methyl methacrylate for the V190A mutant dropped to around 4 M, similar to the value for wt using 2-butanol (data not shown). Saturation was now achievable. This illustrates the importance of alcohol inhibition. The alcohol competes with the acyl donor for the lipase active site and thus affects the observed apparent K_M value. A less potent inhibitor would lead to a decrease in K_M^{app}.

3.4. Reaction media engineering

Fig. 3 shows the measured relative initial rate for the transacylation of methyl methacrylayte catalysed by *C. antarctica* lipase B as a function of the mole fraction of 1-propanol used in the reaction mixture. The reaction rate varied up to 50 times with an optimal

Table 5
Apparent k_{cat} values for enzymatic transacylation of acrylates from pseudo one-substrate Michaelis–Menten kinetics using 25 mM 1-propanol as acyl acceptor in diisopropyl ether at 30 °C. Corresponding apparent k_{cat} values for 470 mM 2-butanol as acyl acceptor are given within brackets.



Catalyst	k_{cat} [min^{-1}]	k_{cat} [min^{-1}]	k_{cat} [min^{-1}]	k_{cat} [min^{-1}]
CALB wt[a]	460	56 (40)[b]	140	860 (60)[b]
V190A[a]	n.m.[c]	170 (109)[b]	660	2600 (329)[b]

[a] *Candida antarctica* lipase B.
[b] Values within brackets are apparent k_{cat} values using 470 mM 2-butanol as acyl acceptor.
[c] Not measured.

concentration of 1-propanol close to 25 mM (0.2 mol%, the value that was used in the transacylation reactions above). For the more sterically hindered primary alcohol 2-ethyl-1-hexanol and the secondary alcohol 2-butanol, the optimal concentrations were found to be around 4–5 mol.% and the reaction rate varied up to 3 times depending on the alcohol concentration (with a similar profile as the curve shown in Fig. 3, data not shown).

3.5. Factors that affect enzymatic acrylation

As was shown in Sections 3.3 and 3.4 steric hindrance, electronic effects and the nature of the acyl acceptor affects enzymatic acrylation. Poor substrate binding can be beneficial for the value of k_{cat} if the transition state for the weak binding substrate is unaffected. This so-called ground state destabilisation (GSD) and circe effect (the case where one part of the substrate provide binding energy for the distortion of another part) has been widely discussed in the literature [26], for example in the context of the source of the rate enhancement for orotidine-5-monophosphate decarboxylase (EC 4.1.1.23) [27,28]. Substrate binding energies ΔG_b and activation energies $\Delta G^{\ddagger}k_{cat}$ and $\Delta G^{\ddagger}k_{cat}/K_M$ for the lipase catalysed transacylation of the different acrylates relative to methyl propionate are shown in Table 6. It can be concluded that the transition state for the different acrylates experience different amount of stabilisation. Ground state destabilisation was concluded to be of less importance.

The influence of electrostatics on enzymatic acrylation was analysed by making the CALB mutation D134N. This mutation changed the charge distribution in the active site around the acrylate substrate. The effect of this mutation was an increased relative specificity of methyl propionate compared to methyl acrylate by a factor of 2. The absolute rate, corrected for the number of active sites, dropped around 2-fold for methyl propionate and 10-fold for methyl methacrylate compared to the corresponding rates for CALB wt. This indicates that electronic effects may be important. Similarly, the idea behind the CALB I285W mutation was to introduce π-stacking. However no rate enhancement for the transacylation of methyl methacrylate was observed.

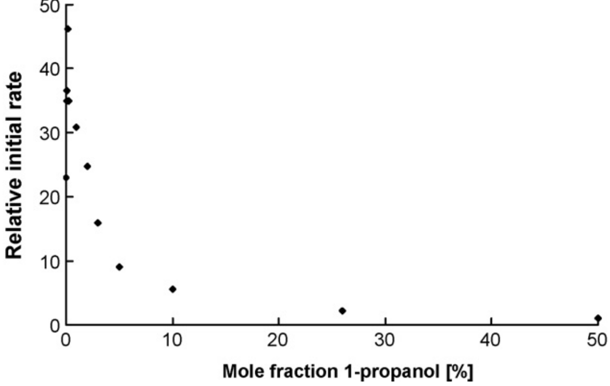


Fig. 3. Relative initial rate of CALB wt-catalysed transacylation of methyl methacrylate as a function of the mole fraction of acyl acceptor (1-propanol).

Table 6
Activation energies and binding energies relative to methyl propionate for *Candida antarctica* lipase B wt calculated from the data given in Section 3.3 at 30 °C.

	$\Delta\Delta G^{\ddagger}k_{cat}$ [kJ/mol][a]	$\Delta\Delta G^{\ddagger}k_{cat}/K_M$ [kJ/mol][b]	$\Delta\Delta G_b$ [kJ/mol][c]
O, H, OMe	3.1	5.5	−2.8
O, Me, OMe	8.4	12.6	−5.1
O, Cl, OMe	6.1	3.2	1.8

[a] Activation energies relative to methyl propionate based on apparent k_{cat} values using 25 mM 1-propanol as acyl acceptor.
[b] Activation energies based on apparent k_{cat}/K_M values using 25 mM 1-propanol and relative to methyl propionate.
[c] Relative substrate binding energies compared to methyl propionate based on apparent K_M values.

The nature and concentration of the acyl acceptor had a big impact on the rate for the enzyme catalysed reaction. For the secondary alcohol 2-butanol concentrations up to 470 mM could be used without significant alcohol inhibition. For 1-propanol, the optimal concentration was 25 mM. In the literature, 2-octanol was found to have an inhibition constant 10 times higher compared to 1-octanol in CALB-catalysed transacylation in heptane [13].

4. Conclusions

Lipases have not evolved to deal with planar substrates such as acrylates. The poor binding of acrylates is accompanied with a decrease in rate. Clearly, sterical and electronic effects contribute to the different amount of TS stabilisation experienced by the different acrylates.

There are several potential ways of increasing reaction rates. Elevated temperature is one, catalyst design is another and optimising the reaction media is a third. Rational enzyme design is competitive with directed evolution approaches in the case of improving enzyme activity for methyl methacrylate. Although potential screening protocols exist like the Purpald method [29], the expected increase in rate of created positive mutants lie within

the error margin of the screening protocol. Our rational design approach was successful and resulted in a CALB V190A mutant with a k_{cat} 3-fold higher than CALB wt for the transacylation of methyl methacrylate. For industrial purposes k_{cat} values are of higher interest than k_{cat}/K_M [30].

The importance of the reaction media was shown by the fact that the reaction rate of lipase catalysed transacylation of methyl methacrylate varied up to 50 times with the concentration of 1-propanol. The combination of enzyme rational design and reaction media engineering can thus lead to additional improvements.

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