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Lipase chemoselectivity towards alcohol and thiol acyl acceptors in a transacylation reaction

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ABSTRACT

The lipase chemoselectivity towards an alcohol and a thiol was investigated for the two lipases *Candida antarctica* lipase B (*Ca*lB) and *Rhizomucor miehei* lipase (*Rm*l). Hexanol and hexanethiol were used as acyl acceptors in a transacylation reaction with ethyl octanoate in cyclohexane. *Ca*lB showed the highest chemoselectivity ratio $(k_{cat}/K_M)_{OH}/(k_{cat}/K_M)_{SH}$, of 88,000 while the ratio for *Rm*l was 1200. That could be compared with the ratio, k_{OH}/k_{SH}, of 120 for the non-catalyzed reaction. Thus, the enzyme contribution to the chemoselectivity between hexanol and hexanethiol was 730 for *Ca*lB and 10 for *Rm*l. High K_M values displayed towards hexanethiol (above 1.8 M) were the largest contribution to the selectivity. No saturation was achieved. The K_M values were more than two orders of magnitude higher than those of hexanol.

1. Introduction

The catalytic properties of triacylglycerol lipases have been extensively explored and they are currently used as efficient catalysts in a number of industrial processes [1–3]. In aqueous solutions they hydrolyse ester bonds, while in an organic environment ester bonds are synthesised. Their ability to perform regioselective, enantioselective and chemoselective catalysis is of outermost synthetic interest. This is used in many areas, like synthesis of chiral drugs [4], kinetic resolution of alcohols, acids, esters and amines [5,6] and regioselective synthesis of sugar esters [7]. In polymer science, polyester synthesis using lipases has been documented as an efficient route of making polyesters by polycondensation or ring-opening polymerisation (ROP) reactions using various different substrates/monomers [8–11]. End-group functionalization of polymers is of great importance for making further complex polymer structures and architectures such as branched and cross-linked polymers [12]. Using the inherent selectivity displayed by lipases, simple one-pot routes are available for making functional and chiral polymers [13]. Thiol as a functional group has many interesting characteristics; it may undergo oxidation, acylation, alkylation and form complexes with many heavy metals [14,15]. Further, thiols can react with a variety of enes in a photo-initiated radical reaction (thiol-ene reaction). In polymer science, this is used as an efficient method for making polymer networks [16,17]. Synthesis of thiol functionalized polymers is traditionally performed using a protection group on the thiol [18,19]. We have previously reported on the synthesis of thiol end-functionalized polyesters by the chemoselective *Candida antarctica* lipase B in an one-pot reaction without protection chemistry [20–22]. Mercapto-alcohols were used as initiators in the ROP of lactones, giving thiol functionalized polymers in high yields. The selectivity displayed by the lipase favoured the hydroxyl group compared with the thiol group.

The use of alcohols as acyl acceptors in lipase catalyzed acyl transfer reactions is well documented. A number of V_{max} and K_M values for several lipases have been reported, even though k_{cat} values based on active-site titration are rare. However, the corresponding kinetic data for thiols are very limited [23]. Further, there are many enantiomeric ratios (E) reported for lipases, while chemoselectivity data for lipases are not available in literature. In order to document the chemoselectivity displayed by lipases towards alcohols and thiols, we here report on the selectivity displayed by the two lipases *C. antarctica* lipase B and *Rhizomucor miehei* lipase towards hexanol and hexanethiol as acyl acceptors in acyl transfer reactions.

2. Experimental

2.1. Materials

Immobilized lipases were bought from Sigma–Aldrich; *C. antarctica* lipase B (*Ca*lB) in form of Novozym 435 and *R. miehei* lipase (*Rm*l) in form of Lipozyme. The water activity of the two enzyme preparations was set to 0.11, using saturated LiCl in a desic-

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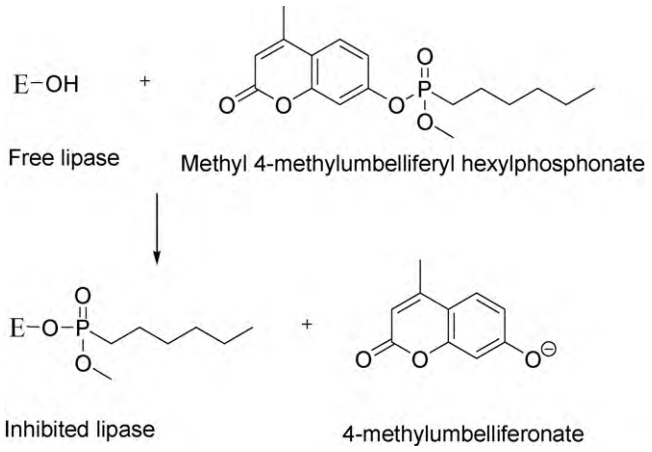


Scheme 1. Inhibition of lipases using methyl 4-methylumbelliferyl hexylphosphonate. The scheme illustrates the formation of the covalently modified active serine as a stable phosphonate intermediate. The 4-methylumbelliferonate is formed in a 1:1 ratio to active enzyme.

cator. All solvents and substrates were dried over molecular sieves (3 Å) for at least one week before use.

2.2. Methods

2.2.1. Active-site titration

Immobilized lipase (10–20 mg) was mixed with 1 ml acetonitrile and methyl 4-methylumbelliferyl hexylphosphonate was added (25 μM) which was 3–40 times the lipase concentration. Acetonitrile was used as solvent due to the insolubility of the active-site inhibitor, methyl 4-methylumbelliferyl hexylphosphonate, in cyclohexane. In order to totally inhibit the lipase, an addition of 1% water to the solvent was needed. Controls with only inhibitor and only lipase were treated in the same way to get the background hydrolysis of the inhibitor and the fluorescence from the carrier. All samples were incubated in darkness at 20 °C. Aliquots (100 μl) from the inhibition reaction (during four days) was added to a cuvette containing 900 μl buffer (100 mM Tris–HCl, pH 8, 1 mM $CaCl_2$). The active-site concentration was determined from the fluorescence intensity (Ex. 360 nm, Em. 445 nm) of the leaving group 4-methylumbelliferonate (see Scheme 1). A standard curve of 4-methylumbelliferone was prepared freshly. All measurements were performed on a Perkin Elmer LS50B fluorescence spectrophotometer. After fluorescence measurements, the immobilized lipase was filtered off and allowed to dry at 0.11. Test reactions (0.016 M hexanol and 0.5 M ethyloctanoate in cyclohexane using decane as internal standard) were performed to determine the activity difference between the incubations with and without inhibitor. This was done to determine the fraction of inhibited enzyme.

Table 1
Active-site titration using methyl 4-methylumbelliferyl hexylphosphonate.

Lipase	Amount active lipase on the carrier	
	nmol/g carrier	weight (%)
Novozym 435 (*Ca*lB)	1,000	3.3
Lipozyme (*Rm*l)	35	0.14

2.2.2. Acylation of hexanol and hexanethiol

Typically 2–20 mg immobilized lipase in 3 or 6 ml cyclohexane containing hexanol (0.001–0.1 M) or hexanethiol (0.1–1.8 M) and decane as internal standard were allowed to stir for 5 min. The reaction was started by adding 0.5 M ethyl octanoate and was performed at 20 °C. All substrates and solvent used were dried with molecular sieves (3 Å). Samples were withdrawn and filtered through glass-wool. Analysis was performed on a Hewlett Packard 5890 Series II gas chromatograph with a Chrompack CP-SIL 5CB column (25 m × 0.32 mm). Inlet and detector temperatures were set at 250 °C. The temperature program started at 40 °C for 5 min, increased by 1 °C/min to 55 °C, then by 5 °C/min to 150 °C and finally by 10 °C/min to 300 °C, where it was kept for 5 min. The retention times were hexanol 17.5 min, hexanethiol 21.7 min, decane 27.6 min, ethyl octanoate 34.5 min, hexyl octanoate (alcohol product) 43.1, and S-hexyl thiooctanoate (thiol product) 45.4 min.

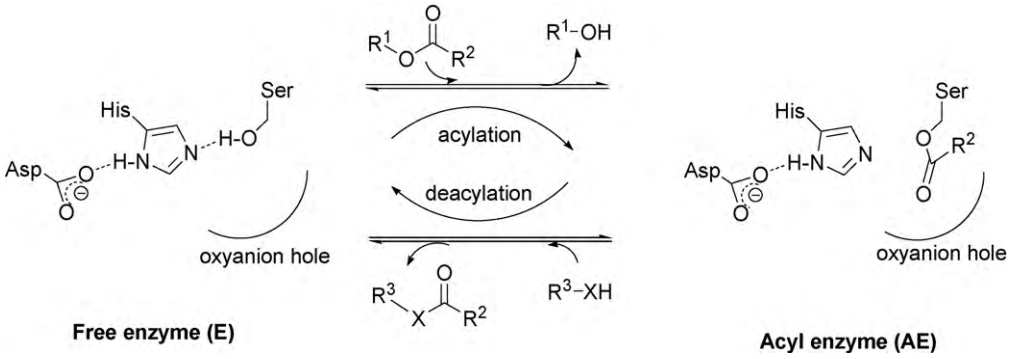
3. Results and discussion

3.1. Active site titration of immobilized lipases in organic solvents

The inhibition of the immobilized lipase preparations, Novozym 435 (*Ca*lB) and Lipozyme (*Rm*l), using the inhibitor methyl 4-methylumbelliferyl hexylphosphonate is illustrated in Scheme 1 [24,25]. The amount of active lipase differed greatly between the enzyme preparations, 3.3 wt% on Novozym 435 (*Ca*lB) and 0.14 wt% on Lipozyme (*Rm*l) (Table 1). The inhibition was slow compared to free *Ca*lB in aqueous media (unpublished data). Plateau values were achieved after four days inhibition. By then >99% of the Novozym 435 (*Ca*lB) and 91% of the Lipozyme (*Rm*l) preparations had been inhibited.

3.2. Acyl transfer reactions

The mechanistic route for lipase catalyzed acyl transfer reactions is illustrated in Scheme 2. With hexanol as acyl acceptor both lipases, *Ca*lB and *Rm*l, displayed saturation kinetics. The apparent



Scheme 2. Lipase catalyzed transacylation reactions using hexanol (X = O) or hexanethiol (X = S) as acyl acceptors.

Table 2
Apparent kinetic constants for the acyl transfer using 0.5 M ethyl octanoate as acyl donor and 0.001–0.1 M hexanol or 0.1–1.8 M hexanethiol as acyl acceptor in cyclohexane.

Lipase	$(k_{cat}/K_M)^{app}$ (s^{-1} M^{-1})	k_{cat}^{app} (s^{-1})[a]	K_M^{app} (M)[a]
*Ca*lB			
Hexanol	710	14 ± 9	0.019 ± 0.002
Hexanethiol	0.0081[b]		>1.8
*Rm*l			
Hexanol	16,000	130 ± 10	0.0084 ± 0.002
Hexanethiol	13[b]	–	>1.8

[a] Non-linear regression of Michaelis–Menten equation.
[b] Calculated from rates as a function of substrate concentrations far below K_M.

Table 3
Chemoselectivity between hexanol and hexanethiol in an acyl transfer reaction with ethyl octanoate.

Lipase	Chemoselectivity	Ratios relative to uncatalyzed
$(k_{cat}/K_M)_{OH}/(k_{cat}/K_M)_{SH}$		
*Ca*lB	88,000	730
*Rm*l	1,200	10
k_{OH}/k_{SH}		
Uncatalyzed	120[a]	1

[a] k_{OH}/k_{SH}, background reactions with no enzyme using vinyl octanoate as acyl donor. No product was detected within ten days of reaction using ethyl octanoate as acyl donor.

kinetic parameters k_{cat} and K_M were determined (Table 2). In contrast, neither of the lipases were saturated when using hexanethiol as acyl acceptor. For both lipases the reaction rate increased linearly with hexanethiol concentrations up to 1.8 M and consequently their K_M values towards the thiol were above that concentration. Concentrations above 1.8 M hexanethiol were not used since it would alter the reaction conditions severely. Thus, only apparent k_{cat}/K_M values from the slope of the Michaelis–Menten plot could be derived for hexanethiol, and these were found to be 0.0081 s^{-1} M^{-1} for *Ca*lB and 13 s^{-1} M^{-1} for *Rm*l. Based on the amount of active lipase, *Rm*l was more efficient in the acylation of both hexanol (23 times higher k_{cat}/K_M) and hexanethiol (1600 times higher k_{cat}/K_M), compared with *Ca*lB.

Both lipases displayed K_M-values towards hexanethiol that were more than two orders of magnitude higher than for hexanol (Table 2). Causette et al. reported that *Rm*l displayed an apparent K_M for butanthiol in hexane of 1.85 M [23]. Lipases are hydrolases that cleave ester bonds in triacylglycerols with use of water. It has been found in several lipases, i.e. *Rm*l and *Ca*lB, that they have an amphiphilic active-site with a hydrophobic part for lipid binding and a hydrophilic part, like a water channel, for water binding [26–28]. We hypothesize that the hydrophilic site around the active serine in lipases is the reason for the high K_M values found for thiols. Thiols are more hydrophobic than alcohols. The transfer free energy (ΔG^{tr}) for thiols when transfered from cyclohexane to water are typically 15–20 kJ mol^{-1} higher than for the corresponding alcohols [29,30]. This means that the partitioning from the reaction phase (cyclohexane) to the catalyst phase (hydrophilic water channel site) will be less favourable for thiols than for alcohols. Based on this simple model 2–3 orders of magnitude higher K_M-values for thiols as compared with alcohols could be predicted.

3.3. Chemoselectivity

The chemoselectivity ratio $(k_{cat}/K_M)_{OH}/(k_{cat}/K_M)_{SH}$ was calculated to be 1200 for *Rm*l and 88,000 for *Ca*lB (Table 3). To quantify the chemoselectivity of the un-catalyzed reaction, the activated ester of vinyl octanoate was used, since the spontaneous reaction with ethyl octanoate could not be detected within ten days. The ratio of the rate constants, k_{OH}/k_{SH}, of the uncatalysed reaction with vinyl octanoate was 120. Thus, the enzymatic contribution to the chemoselectivity ratio was 10 for *Rm*l and 730 for *Ca*lB (Table 3). For both lipases, the largest contribution to the chemoselectivity was the higher K_M values displayed towards hexanethiol compared with hexanol. The K_M ratio was more than two orders of magnitude. For *Ca*lB the selectivity may entirely be a K_M effect, as no saturation was achieved with the thiol. This is in line with results using a thioester as acyl donor in transacylation reactions giving similar k_{cat}-value as the corresponding oxy ester but a 15-fold higher K_M [31].

4. Conclusion

The chemoselectivity towards hexanol and hexanethiol displayed by two immobilized lipase preparations, *C. antarctica* lipase B (*Ca*lB) in form of Novozym 435 and *R. miehei* lipase (*Rm*l) in form of Lipozyme, was investigated. The enzyme contribution to the chemoselectivity ratio between hexanol and hexanethiol $(k_{cat}/K_M)_{OH}/(k_{cat}/K_M)_{SH}$, was 10 for *Rm*l and 730 for *Ca*lB. Most of the selectivity was a result of high K_M values for hexanethiol (>1.8 M), which were at least two orders of magnitude higher than those for hexanol.

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