Enhancement of *Candida antarctica* lipase B enantioselectivity and activity in organic solvents

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The enantioselectivity and catalytic activity of Novozym 435® [*Candida antarctica* lipase B (CALB)] in organic solvents was found to dramatically increase upon the addition of a non-reactive organic base, such as Et_3N, to the reaction system.

It has been shown that the unusual microenvironment of enzymes in organic solvents can affect a number of parameters, including the degree of protein hydration,[1,2] secondary structure,[3] the susceptibility of the protein to inactivation and variations in the ionisation state[4] of side-chain residues. Frequently, these differences have been shown to result in interesting changes in the enzymes, including reversal of substrate specificity and changes in stereoselectivity, although the underlying reasons remain poorly understood.

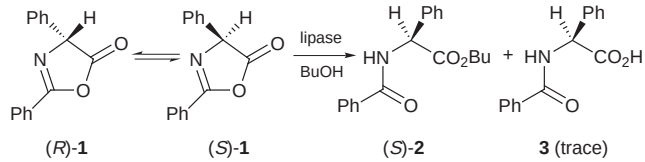
It is commonly accepted that the best predictor of enzyme catalytic activity in low water organic media is thermodynamic water activity (a_w).[1]‡ Over the past few years although much has been reported on enzyme enantioselectivity in organic media there are as yet no predictive rules available. Crude lipase preparations have proved to be simple and effective biocatalysts for kinetic resolutions, *e.g.* chiral carboxylic acids and alcohols. However, the low purity of these preparations (presence of other lipases and competing hydrolases) can, in specific reactions, lead to low and unpredictable enantioselective behaviour. This effect can be compounded when using organic solvents, due to the effect of different solvent properties on catalytic activity.

The starting point for the work described herein was the lipase (Lipozyme® *Mucor miehei*) catalysed dynamic resolution of 4-substituted oxazol-5(4*H*)-ones, a reaction we have previously employed for the synthesis of enantiomerically pure (*S*)-L-*tert*-leucine.[5] It was previously found that the modest enantioselectivity in toluene (*ca.* 68% ee) could be enhanced (*ca.* 97% ee) by the addition of a catalytic amount of Et_3N to the reaction; the role of Et_3N is not to facilitate racemisation of the substrate.

We decided to investigate this effect in more detail by using a commercially available immobilised lipase,§ Novozym 435 (*Candida antarctica* lipase B[6] (CALB), since a larger substrate range could be tested with this enzyme. The catalytic activity and enantioselectivity of the alcoholysis of (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one **1** using butan-1-ol as the nucleophile (Scheme 1) was monitored¶ under a range of reaction conditions, including controlled water activity. Hydration was controlled by equilibrating‖ enzyme and solvent with the appropriate saturated salt solution[7] of known thermodynamic water activity a_w. Therefore a low a_w system will be one in which the solvent is poorly hydrated and the enzyme, similarly, has a low level of hydration, and at high a_w (*e.g.* 0.97) the solvent is near water saturation and the enzyme is fully hydrated (as would be found in an aqueous system). Table 1 shows the effect of hydration on the initial catalytic rate and enantioselectivity, in three different solvents, n-hexane, toluene and MeCN, either with or without Et_3N.**

It can immediately be seen that the lipase-catalysed reaction is very sensitive to water activity. The addition of a non-reactive organic base,†† Et_3N, to the reaction enhances significantly both the enantioselectivity and catalytic activity of the enzyme. Even low levels of hydration, present in the more nonpolar solvents such as n-hexane and toluene, are detrimental to the overall catalytic performance of CALB. We find that generally for optimum yield and enantioselectivity, both the enzyme and solvent should be rigorously dried prior to addition of Et_3N. We were interested to see if addition of Et_3N to a reaction already in progress and of poor enantioselectivity, could reverse this effect. As can be seen from Fig. 1, the addition of Et_3N after 140 min immediately results in enhanced catalytic rate and enantioselectivity.

In order to examine the generality of the effect of Et_3N we investigated a second reaction, namely the CALB-catalysed



Scheme 1

Table1 Effect of water activity on initial catalytic rate[a,b] and enantiospecificity as a function of hydration, with and without Et_3N

		No Et_3N		Et_3N	
Solvent[c]	a_w	Initial rate/nmol min^{-1} mg^{-1}	Ee (%)	Initial rate/nmol min^{-1} mg^{-1}	Ee (%)
n-hexane	~0 (anhydrous)	26 (± 1.5)	85 (± 3)	30 (± 1.5)	90 (± 3)
n-hexane	0.69	4 (± 0.5)	55 (± 2)	20 (± 1)	87 (± 3)
n-hexane	0.97	1.5 (± 0.15)	30 (± 5)	18 (± 0.9)	80 (± 5)
toluene	~0	15 (± 0.8)	85 (± 4)	27 (± 1.5)	93 (± 3)
toluene	0.22	3	61 (± 6)	17 (± 1)	95 (± 2)
MeCN[d]	~0	15	>99	10	97 (± 2)
MeCN[d]	0.1 (0.5% v/v H_2O)	NR[e]	—	5 (± 0.3)	90 (± 4)
MeCN[d]	0.4 (2% v/v H_2O)	NR[e]	—	NR[e]	—

[a] Initial rate for (*S*)-butyl ester enantiomer **2**. [b] Results reported are the average of three separate measurements. [c] Note ‖. [d] Ref. 8. [e] No reaction.

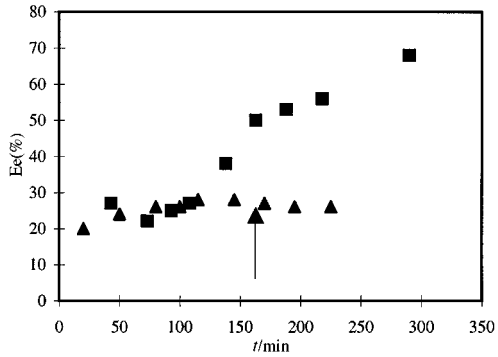


Fig. 1 Effect of Et_3N on ee. Reactions A (▲) and B (■) were carried out under identical conditions (a_w = 0.69). At t = 140 min, 14 mol% Et_3N was added to reaction B (arrow).

reaction between 1-phenylacetoxy-2-methylcyclohexene and butanol yielding 2-methylcyclohexanone and butyl phenylacetate.[9,15] Using n-hexane (a_w = 0) and MeCN (0.5% H_2O, a_w = 0.1) as the solvents, we observed that the addition of Et_3N to the solvent resulted in a dramatic increase in the catalytic activity. An approximate 200-fold increase in activity was observed in MeCN (a_w = 0.1) and a 700-fold one for that in n-hexane (a_w = 0.97). The higher activity found in n-hexane is presumably due to a more intimate contact between the enzyme and Et_3N in a more nonpolar environment. Similarly, the activation effect for (±)-2-phenyl-4-benzyloxazol-5(4*H*)-one ring-opening in MeCN is similar to that described above and is expected to be a result of less Et_3N adsorption to the enzyme in MeCN.

The ability of organic bases to increase the enantioselectivity of lipase-catalysed reactions in water-saturated organic solvents has previously been reported.[10–13] In some cases[11,12] this effect has been attributed to the formation of an ion-pair between the base and any by-product acid. Using electrospray ionisation mass spectrometry (ESI-MS)‡‡ we have detected the formation of carboxylic acid **3** during the course of the oxazolone reaction at intermediate to high water activities (*e.g.* a_w = 0.69–0.97). We have also found that addition of acid **3** to an already hydrated system results in loss of activity, which can be fully recovered upon addition of an organic base, presumably *via* formation of an ion pair. Ion pair formation is observed in both low and high dielectric non-hydrogen bonding solvents such as n-hexane and MeCN. In a high dielectric, non-hydrogen bonding solvent such as MeCN, where the acid was found to be more soluble, we find experimentally that dissolution of acid **3** in n-hexane and MeCN occurs upon addition of Et_3N, thus removing acid from the immediate microenvironment of the enzyme. However, the enhancement of catalytic performance and enantioselectivity for rigorously dried samples, and those of low water activity (a_w < 0.7) where we find no evidence for hydrolysis over the course of the initial rate measurement, cannot be explained in terms of hydrolysis products affecting enantioselectivity, since for an unrelated substrate, an activating effect on the catalytic activity has been demonstrated.

The addition of co-solvents, such as DMF and DMSO, was found to solubilise the acid and thus it was anticipated that they would perform a similar role to Et_3N in removing any acid from the immediate vicinity of the enzyme. Both DMF and DMSO were chosen as additives to the bulk organic solvent (toluene at a_w = 0.22). Although both DMF and DMSO increased the enantioselectivity of the reaction to 85% ee, there was no significant effect on the catalytic rate as found with Et_3N. Since the solvation of the carboxylic acid by these co-solvents occurs by a different mechanism to that of Et_3N, *i.e.* the additives are unable to form ion-pairs, they have limited use in reducing the overall effect.

The role of Et_3N therefore appears to be dual in nature, *i.e.* increasing both the enantioselectivity and catalytic activity of lipase-catalysed reactions. The addition of Et_3N therefore provides an additional strategy for improving the enantioselectivity of lipase-catalysed reactions. We are currently investigating this effect with other lipolytic enzymes.

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Notes and References

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‡ The thermodynamic water activity (a_w) describes the mass action effect of water on hydrolytic equilibria and also describes the partitioning of various water phases that can compete for water binding (ref. 1).

§ Polyacrylamide gel electrophoresis of CALB desorbed from the solid support exhibited a single band corresponding to the reported molecular weight of CALB (33 KDa) (ref. 6).

¶ (±)-2-Phenyl-4-benzyloxazol-5(4*H*)-one **1** (0.16 mmol) was placed in a 4 ml screw top vial together with the solvent, (either anhydrous or hydrated), butan-1-ol (0.24 mmol, 1.5 equiv.) CALB (40 mg) and Et_3N (14 mol%). The reaction vial was shaken at 250 rpm on a rotary shaker at 37 °C and the progress and ee (%) of the reaction were monitored by chiral HPLC (Chiralcel-OD, 250 × 4.6 mm, Mallinckrodt Baker, n-hexane–Pr^iOH (90:10 v/v), UV detection λ = 254 nm).

‖ *Candida antarctica* lipase B (CALB) was received as an immobilised preparation (Novozym 435, Boehringer Mannheim, Germany) and was dehydrated over P_2O_5 (at room temp.) for 2–3 days. Rehydration of dried lipase to the desired water activity (a_w) was carried out using saturated salt solutions (equilibration period 48–72 h). (±)-2-Phenyl-4-benzyloxazol-5(4*H*)-one **1** was stored over P_2O_5 at 0 °C; anhydrous solvents were stored over freshly reactivated 3 Å or 4 Å molecular sieves. The water content of dried solvents was measured using Karl Fischer water titration (ref. 15) and found to be < 0.001 wt%. Solvents were hydrated separately from the enzyme using the same water equilibration procedure as described above, approximately 24 h before use.

** Control reactions showed that no detectable ester (as judged by HPLC) was formed in the absence of enzyme, either with or without Et_3N, over a 48 h analysis period.

†† Other organic bases give very similar results to Et_3N, *e.g.* DABCO and lutidine. Insoluble inorganic bases, *e.g.* $KHCO_3$ and K_2CO_3, had no effect and did not result in the high catalytic rate and enantioselectivity observed with the soluble organic bases.

‡‡ Electrospray ionisation mass spectrometry (ESI-MS) and atmospheric chemical ionisation (APCI) were performed on a Micromass Platform II spectrometer (cone voltage 20 V).

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