'Immunization' of Lipase against Acetaldehyde emerging in Acyl Transfer Reactions from Vinyl Acetate

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Immobilization of *Candida cylindracea* lipase by covalent linkage involving the ε-amino residues of lysine leads to a five-fold increase of selectivity which is entirely preserved against deactivation caused by acetaldehyde, an unavoidable by-product in acyl transfer reactions with vinyl acetate.

Lipase-catalysed acyl transfer performed in organic solvents at low water content has proved to be a valuable technique for the biocatalytic resolution of racemic or asymmetrisation of prochiral primary and secondary alcohols.[1] Among the advantages offered by this method such as increased overall yields, enhanced stability of enzymes,[2] lack of water-dependent side reactions,[3] altered specificity of enzymes[4] and the successful transformation of highly lipophilic solid substrates which in general cannot be transformed in an aqueous medium,[5,6] it was generally assumed that reuse of the enzyme after its easy recovery by filtration offered another advantage.[7]

Table 1 Optical purity of products

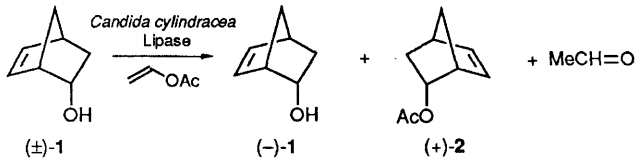
Entry	Enzyme	System	Relative rate (%)	Conversion (%)	E.e. of (−)-**1** (%)	E.e. of (+)-**2** (%)	E[13]
1	Native	Standard[a]	38	51	51.4	66.5	8
2	Recovd. from entry 1	Standard[a]	4	25	11.0	54.0	4
3	Treated with MeCH=O	Standard[a]	4	19	5.1	22.2	1.6
4	Native	Standard[b]	100	58	99.4	72.0	34
5	Native	Standard + dimedone[c]	43	48	53.1	71.3	11.5
6	Native	Standard + aminoguanidine·HCl[c]	6	66	96.8	72.9	10.8
7	Native	Standard + $NaHSO_3$[c]	23	54	66.0	73.5	17.5
8	Immobilized	Standard[d]	47	46	62.2	91.3	42
9	Recovd. from entry 8	Standard[d]	29	48	66.0	91.7	42
10	Recovd. from entry 9	Standard[d]	15	44	59.1	91.8	42

[a] Standard conditions: a mixture of (±)-**1** (1 g, 9 mmol), lipase (Amano AY-30, 200 mg) and vinyl acetate (10 ml) was shaken at 20 °C with 200 r.p.m. E.e. = enantiomeric excess. [b] Standard conditions were used with 500 mg of lipase. [c] Aldehyde-quencher (9 mmol) was added to the standard system. [d] Lipase immobilized onto VA-Epoxy Biosynth[20]‡ with an activity corresponding to that of the standard conditions was used.

During the optimization and scale-up of a recently published *Candida cylindracea* lipase catalysed resolution of a bicyclic secondary alcohol[5] using vinyl acetate as the acyl donor, we encountered unexpected difficulties. In contrast to previously published work,[7–10] we observed a significant decrease in both activity and enantioselectivity upon repeated use of the lipase. Aiming at elucidating the destructive mechanism and in an effort to find possible methods to avoid it, we initiated this study on lipase from *C. cylindracea*.† *endo*-Bicyclo[2.2.1]hept-5-en-2-ol (±)-**1** was chosen as the model compound, since this substrate was previously resolved with fair but still improvable selectivity (E = 28) *via* lipase-catalysed hydrolysis of the corresponding esters;[11] furthermore its optical purity can accurately be determined by GLC-analysis.[12] This latter fact is particularly important in view of the problems which have been encountered in the determination of an enzyme's selectivity, in particular the enantiomeric ratio (E),[13] if values above $E \approx 20$ are to be determined.[14]

As shown in Table 1, the resolution of the substrate (±)-**1** using native crude lipase from *C. cylindracea* proceeded with low enantioselectivity (entry 1, E = 8). When this lipase sample was recovered and reused in a duplicate run, its selectivity dropped to about half and its activity was almost entirely lost (entry 2). When fresh *C. cylindracea* lipase was pretreated with a solution of acetaldehyde in vinyl acetate with concentrations mimicking the reaction conditions at 50% conversion, a slow reaction rate and poor selectivity were observed from the beginning (entry 3). Furthermore, a higher ratio of enzyme *vs.* substrate corresponding to 50% (w/w) gave better results indicating a link between loss of activity and the stoichiometry of the reaction (entry 4). Both of the latter experiments strongly suggested that the co-product acetaldehyde was the cause for the enzyme's deactivation. Although the deactivating effects of acetaldehyde in this type of reaction were previously assumed,[15] the effects of the molecular sieve which was added to the reaction medium were not further investigated.[9,16]

Acetaldehyde is known to act as an alkylating agent on enzymes by forming Schiff's-bases in a Maillard-type reaction[17] particularly on terminal amino residues of lysine.[18] As depicted in Scheme 2, a positive charge is removed from the enzyme's surface during the course of this reaction leading to possible deactivation.



Scheme 1 Enzymatic resolution of (±)-**1**

Since the Schiff's-base formation is reversible, attempts were undertaken to 'reactivate' the completely deactivated enzyme by exposure to aldehyde-trapping agents such as n-butylamine, hydrogen sulphite and 5,5-dimethylcyclohexane-1,3-dione (dimedone) in order to recover its activity. Although the 'reactivated' enzyme samples showed some recovered activity in each case, the recovery in selectivity was negligible.

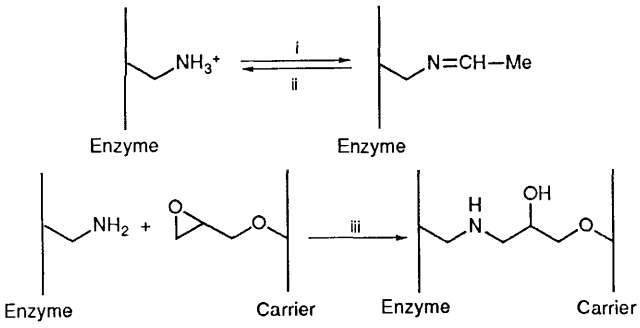
Next, attempts were made to avoid the deactivation from the beginning of the reaction by addition of aldehyde-quenching reagents to the reaction medium (entries 5–7). Here the effects were more pronounced: although a depletion of the reaction rate could still not be avoided, the selectivity was markedly enhanced when compared to the results which were obtained with untreated native lipase. Hydrogen sulphite was the best aldehyde-quencher (E >17, entry 7).

Our final approach was aimed at the modification of the enzyme itself by selectively blocking its reactive groups, in particular the ε-amino functionalities of lysine. From a number of methods available for the selective chemical derivatization of terminal amino functionalities,[19] immobilization *via* irreversible alkylation by epoxy-activated macroscopic carriers[20] was chosen, which proceeds selectively under mild conditions and with high retention of activity. The so-formed covalent linkages are primarily achieved by *N*-alkylation of ε-amino groups of lysine yielding a highly desirable retention of charge on the enzyme's surface. Thus, secondary amino functionalities are obtained from primary ones which makes the enzyme 'immune' towards the formation of Schiff's-bases with aldehydes (see Scheme 2).

Performing the standard model reaction with *C. cylindracea* lipase immobilized onto VA-Epoxy Biosynth[20]‡ resulted in a greatly stabilized activity with a simultaneous five-fold enhanced selectivity (E >40) compared to the properties of the native lipase. Furthermore, this increased selectivity could be entirely preserved in repetitive use over three consecutive

† Lipase AY-30 from *Candida cylindracea* (Amano Pharm. Co. Ltd., Japan) was used as received.

‡ Epoxy-activated polymer support VA-Epoxy-Biosynth™ from Riedel de Haen (FRG), product no. 39354.



Scheme 2 Alkylation and immobilization of enzyme. *Reagents and conditions*: i, MeCH=O; ii, H_2O; iii, buffer, pH = 7, 3 days, room temp.

runs (entries 8–10). It is noteworthy that immobilisation of the enzyme by simple adsorption onto Celite[21] did not lead to any analogous improvement.

In conclusion this study shows that alkylation of terminal lysine residues by covalent immobilization led to a markedly enhanced enantioselectivity of *C. cylindracea* lipase in acyl transfer reactions using vinyl acetate as acyl donor. Furthermore, in contrast to the native lipase, the enantioselectivity of the immobilized enzyme was totally immune against deactivation reactions caused by acetaldehyde. An extended study on the use of chemically derivatized enzymes is in progress.

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