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Organic co-solvents restore the inherently high enantiomeric ratio of lipase B from *Candida antarctica* in hydrolytic resolution by relieving the enantiospecific inhibition of product alcohol

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Abstract

The enantiomeric ratio in the hydrolysis of racemic 3-chloro-1-phenylmethoxy-2-propyl butanoate with lipase B from *Candida antarctica*, CALB, is raised from E=50 to E=180 upon addition of acetone to the aqueous medium. This co-solvent effect is now explained as an enantiospecific inhibition of the lipase by the liberated alcohol. In the range from 0 to 30% acetone, the effect correlates with the increased solubility of the alcohol in the reaction medium. Free and immobilized CALB show similar behaviour.

1. Introduction

In many instances, otherwise interesting combinations of enzymes and racemic substrates show a low enantiomeric ratio (E-value) in kinetic resolutions. Typically, E=10 has been recognized as a lower limit for the feasibility of enzyme-catalyzed kinetic resolution processes. High E-values (E=100) are required for practical applications designed to give high yields of enantiopure products. Several options are available to improve the enantiomeric ratio of a combination of interest, e.g. change of protecting groups, change of co-reactant (i.e. acyl donor in lipase-catalyzed resolutions), change of medium, change of enzyme preparation (i.e. free or immobilized enzyme). The importance of these factors for the resolution of secondary alcohols derived from glycerol using CALB has been discussed.[1] Among the various possibilities, changes of the reaction medium are most easily implemented.[2]

During investigations of solvent effects in the hydrolysis of the butanoate of 3-chloro-1-phenylmethoxy-2-propanol **1a** using CALB (Scheme 1) we discovered that E increased remarkably on addition of co-solvents. The most significant effects were observed for acetone and *tert*-butanol. The effect

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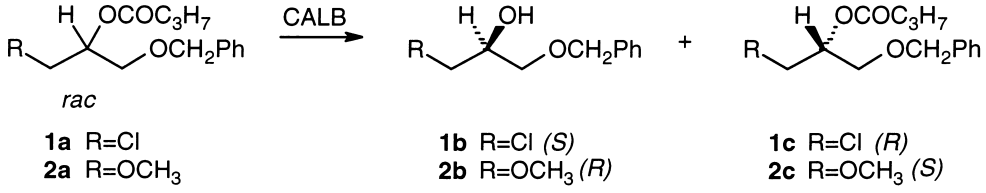
reached a maximum for 30% acetone.[3] In an attempt to explain the observed solvent effect we recorded the ^{1}H NMR spectra of pure CALB in water and with addition of up to 50% acetone. No change in the spectrum was detectable. We therefore concluded that the conformation of the enzyme did not change on addition of acetone. Moreover, when the phenyl protons of racemic 3-chloro-1-phenylmethoxy-2-propanol (*rac*-**1b**) in pure D_2O solution were irradiated, no nuclear Overhauser effect (NOE) was observed on the other protons. However, when dissolved enzyme was added to the NMR solution, an NOE was observed indicating that the correlation time of the substrate had become longer due to interaction with the enzyme.



Scheme 1.

A slight influence on enantioselectivity by addition of co-solvents has previously been observed in lipase catalyzed hydrolysis of the benzoate of 1,2-isopropylidene glycerol. The maximum *ee* of the remaining substrate was observed using co-solvents with low log P-values such as DMSO and dioxane.[4] A significant increase in stereoselectivity ratio (*E*) was observed for hydrolysis of single enantiomers of methyl 2-acetamido-2-phenylacetate using α-chymotrypsin and 25% DMSO as co-solvent.[5] In the resolution of alkane-2- and -3-yl-acetates by hydrolysis catalyzed by *Pseudomonas cepacia* lipase, addition of 40% acetone increased the *E*-values up to five fold.[6] So far no satisfactory explanation has been given for the observed solvent effects which can be both positive or negative with respect to enantioselectivity depending on enzyme, substrate and solvent.

2. Results and discussion

We have re-investigated the influence of co-solvents on the enantiomeric ratio of hydrolysis of **1a** catalyzed by immobilized CALB and confirmed the observation that a maximum *E*-value was reached upon addition of 30% acetone (Table 1). Since data were collected for various extents of conversion and subsequently fitted to relevant equations, the values presented now are more accurate. They differ slightly from those reported previously. For the later work, we found it experimentally practical to use free dissolved enzyme for hydrolysis, since there was a tendency of the substrate to stick to the carrier material. However, measurements performed using both dissolved enzyme and enzyme immobilized on Lewatit gave similar results. Moreover, it was surprising to observe that replacing acetone with 10% hexane, thus introducing an immiscible organic solvent, also increased the *E*-value (Table 1). Further addition of hexane or acetone gave no increase, indicating this value as the maximum intrinsic *E*-value.

A possible explanation of the co-solvent effect is change of solubility of the substrate and/or product. Consequently we measured the solubility of **1a**, **1b**, **2a** and **2b** in relevant solvent systems (Table 2).

When comparing the results with previous and present enantiomeric ratios of hydrolysis, some conclusions may be drawn. Firstly, the solubility of the substrates **1a** and **2a** does not vary significantly. It remains low throughout the solvent series and it may be concluded that the observed solvent effect is not connected with the substrate. Regarding the alcohols produced in the reaction, **1b** and **2b**, the solubility of the latter is high throughout the solvent series. This correlates well with the observed change of *E*-values upon acetone addition.[3] However, the increasing *E*-value upon addition of acetone in the hydrolysis of **1a**

Table 1
E-Values for CALB-catalyzed hydrolysis of **1a** in various solvent systems using immobilized and free enzyme

Solvent system	Immobilized enzyme	Free enzyme
Phosphate buffer, pH 7.2	51	51
10% Acetone	94	n.d.
30% Acetone	158	180
50% Acetone	103	n.d.
10% Hexane	74	154

Table 2
Solubility (μL substrate/mL solvent) of substrates **1a** and **2a** and products **1b** and **2b** in various solvent systems. At a solubility higher than 40 the substrate was considered to be completely dissolved since this concentration is much higher than the amount of substrate used or product formed in the experiments (ca. 10 μL/mL)

	Water	10% Acetone	30% Acetone	10% *t*.-butanol	Hexane
1a	0.03	0.04	0.3	0.04	>1000
1b	7	9	21	>40	63
2a	0.5	0.9	2.7	0.7	n.d.
2b	>40	>40	>40	>40	n.d.

correlates well with an increase of solubility of the produced alcohol (*S*)-**1b**. Thus, the observed solvent effect is connected with low solubility of the liberated alcohol which seems to inhibit the reaction in a specific manner.

Extending this reasoning, addition of (*S*)-**1b** at the start of the reaction should lower the *E*-value. A series of experiments was performed in water/hexane two-phase systems with different amounts of (*S*)-**1b** added at the start of the reaction. In this system, the ee_p-value became difficult to determine and the *E*-value had to be calculated using conversion and ee_s.[7] Since the enantioselectivity of CALB for the hydrolysis reaction of **1a** is high, the degree of conversion had to be determined to a high degree of accuracy. This reaction however, could not be used to determine the exact amount of substrate added to the reaction mixture since the reaction stops at approximately 50% conversion. Lipase from *Candida rugosa* was used displaying low enantioselectivity, to run a 100% conversion to determine the exact amount of substrate in moles per gram.

The *E*-value decreased as more (*S*)-**1b** was added to the reaction mixture, confirming our hypothesis (Fig. 1). Moreover, when racemic alcohol *rac*-**1b** was added the same effect was observed, with a similar magnitude. This observation indicates that both of the enantiomers cause the effect. Thus, our earlier NMR studies (see Introduction) which indicated an interaction between CALB and *rac*-**1b**, were confirmed. Since both enantiomers cause the same specific inhibition it may indicate that the effect does not take place in the active site itself, but rather at some other site in the enzyme.

During the experiments leading to the data in Fig. 1 we encountered an analytical problem since the

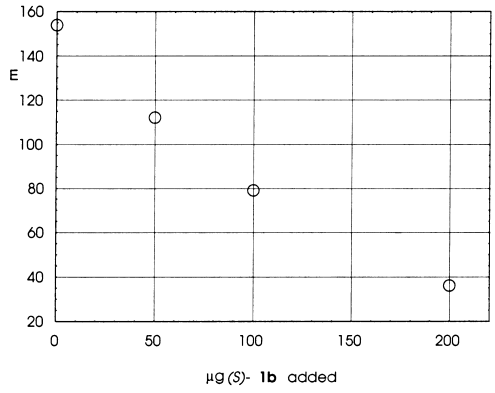


Fig. 1. Effect of addition of (*S*)-**1b** on enantiomeric ratio *E*

product of the hydrolysis was added to the reaction mixture. In order to avoid this problem we also performed the hydrolysis in the presence of a related alcohol, (*R*)-, (*S*)- and racemic 3-chloro-1-phenoxy-2-propanol.[8] Indeed, we found the same specific inhibition, however, to a slightly smaller extent.

3. Conclusion

It has previously been observed that the enantiomeric ratio of the *Candida antartica* lipase B catalyzed hydrolysis of the butanoate of 3-chloro-1-phenylmethoxy-2-propanol increased on addition of 30% acetone. This co-solvent effect is now explained as an enantiospecific inhibition by the liberated alcohol. Addition of either enantiopure (*S*)-**1b** and *rac*-**1b** to the reaction medium leads to a decrease of *E* in hydrolysis. Evidence for the inhibitive adsorption of the alcohol onto the enzyme is provided by ^{1}H NMR spectroscopy. The effect correlates with an increased solubility of the alcohol upon addition of acetone to the reaction medium. Free and immobilized CALB show similar behaviour.

4. Experimental

4.1. Enzymes

Lipase B from *Candida antartica* (CALB) (EC 3.1.1.3), immobilized on Lewatit (Novo-Nordisk, Novozyme SP 435), had specific activity 19 000 PLU/g. Lyophilised CALB (Boehringer-Mannheim, Chirazyme® L-2) had specific activity 1 MU/5.78 g. *Candida rugosa* lipase was purchased from Sigma. All of the solvents were of analytical grade.

4.2. Solubility measurements

Solubility measurements were performed by making saturated solutions of the organic compound in 5 or 20 mL of the different solvent systems, by adding the compound and shaking in a Gyrotory Shaker model G2, 300 rpm, for a minimum of 12 h. The sample tubes were centrifuged for 20 min in an IEC Centra CP8R centrifuge at 3660 rpm. Samples from the supernatant were analyzed on a LiChrosphere 100 RP-18 HPLC column, connected to a Waters pump, and detected on a Hewlett Packard UV/Vis detector at 245 nm (eluent acetonitrile:water, 70:30 or 50:50 v/v). Calibration curves were used to calculate the concentrations.

4.3. Enzymatic hydrolysis

Enzymatic hydrolysis was performed using a Methrom pH-stat equipped with a thermostated reaction vessel at 30°C, pH 7.2 (potassium phosphate buffer, 0.1 M) and the degree of conversion was monitored by addition of 1.002 M NaOH. Substrate (50 μL, accurately measured) was suspended in the solvent system (5 mL) which consisted of buffer and co-solvent (% v/v). Enzyme (25 mg) was added after being dissolved in buffer. Samples (0.25 mL) were withdrawn, extracted with Et_2O, dried over anhydrous $MgSO_4$ and analyzed. For experiments with addition of (*S*)-**1b**, a micropipette was used, 50 μL=50.8 mg.

4.4. Analytical methods

The enantiomeric excess of the substrate (ee_s) was determined by GLC using a Chrompack instrument on a chiral column, CP-Chirasil-dex-CB, supplied by Chrompack. The enantiomeric excess of the liberated alcohol (ee_p) was determined by HPLC using a Waters HPLC system and chiral column, Chiralcel OD-H, supplied by Astec, Whippany, NJ (solvent system hexane:2-propanol, 9:1, flow 0.5 mL/min).

4.5. 3-Chloro-1-phenylmethoxy-2-propanol rac-*1b*

Epichlorohydrin (50 g, 0.55 mol), benzylalcohol (40 g, 0.37 mol) and tetrabutylammonium hydrogen sulfate (3 g) were stirred at 0°C.[9] NaOH (50% w/w, 200 mL) was slowly added and the reaction was stirred for 24 h. Water (180 mL) was then added, and the reaction mixture was extracted with EtOAc. The combined organic phase was washed with saturated NaCl solution and dried over anhydrous $MgSO_4$. The solvent was removed by evaporation under reduced pressure to yield 55 g (90%) of phenylmethyl glycidyl ether. This product (50 g, 0.31 mol) was dissolved in anhydrous THF (250 mL), added to a stirred solution of 0.5 M Li_2CuCl_4 (0.465 mol) in anhydrous THF and stirred under an N_2 atmosphere for 24 h.[10] The reaction was stopped by addition of phosphate buffer (0.05 M, pH 7.0, 500 mL), THF was evaporated under reduced pressure, and the remaining water phase extracted with EtOAc. The combined organic phase was dried over anhydrous $MgSO_4$ and the solvent removed by evaporation under reduced pressure to yield 50 g (81%) of *rac*-**1b**.

4.6. Butanoate of 3-chloro-1-phenylmethoxy-2-propanol *1a*

A mixture of *rac*-**1b** (40.7 g, 0.20 mol) and Et_3N (36 mL, 0.25 mol) in Et_2O (400 mL) was stirred at 0°C. Butanoic chloride (27 mL, 0.25 mol) in Et_2O (200 mL) was added dropwise, and the reaction mixture stirred for 36 h. The formed salt was filtered, the organic phase washed with saturated $NaHCO_3$ solution and the Et_2O removed under reduced pressure. The crude product was distilled twice and afforded the butanoate in excellent purity, yield 34.6 g, 63%.

4.7. 3-Methoxy-1-phenylmethoxy-2-propanol rac-*2* and butanoate rac-*2a*

The synthesis of *rac*-**2b** and the corresponding butanoate *rac*-**2a** have been described previously.[3]

4.8. Batch synthesis of (R)-**1c** and (S)-**1b**

Racemic ester **1a** (5 mL, 4.9 g) was suspended in solvent (30% acetone, 500 mL), immobilized enzyme (1 g) was added and the reaction mixture was stirred for 24 h. Extraction with Et_2O, washing of the organic phase with $NaHCO_3$, drying over anhydrous $MgSO_4$, and separation of the ester and the alcohol by column chromatography (silica gel, acetone:hexane, 5:95) afforded 0.8 g of the ester (*R*)-**1c** (*ee* >99%) and 1.3 g of the alcohol (*S*)-**1b** (*ee*=93%). For physical data ($[\alpha]_D$, NMR, GLC) see previous work.[3,8]

4.9. Enantiomeric ratios

The *E*-values were calculated on the basis of accurate measurements of ee_p and conversion and ee_p and ee_s using two different methods.[11,12]

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