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The *Candida antarctica* Lipase B Catalysed Kinetic Resolution of Seudenol in Non-Aqueous Media of Controlled Water Activity

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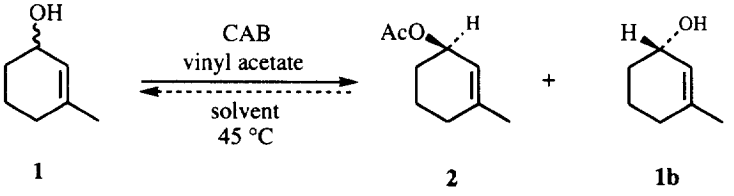
Abstract: For further investigation of the kinetic resolutions in transesterification reactions with the highly enantioselective *Candida antarctica* lipase B, an easy to study model reaction with one typical substrate, the Douglas Fir Beetle pheromone 3-methyl-2-cyclohexen-1-ol (Seudenol), was developed. The influence of the nature of the solvent and thermodynamic water activity was studied. Initial rates showed constant or progressively increasing values with increasing water activity. Enantioselectivity depended on the choice of solvent and descended in most cases with increasing water activity. No general correlations of enantioselectivity or activity with physicochemical constants of the solvent were found. However, at a water activity of 0.11 a tendency toward optimum hydrophobicity (*i.e* log $P \approx 2$) for enantioselectivity was observed. Enantiomeric ratios were in the range 8-32.

The *systematic* study of enzymatic reactions in non-aqueous media is growing rapidly with the occurrence of enzymes, in particular serine-hydrolases, as "on the shelf reagents" in organic chemistry. Numerous useful applications have been developed.[1]

It has been shown that many circumstances influence the final result of an enzymatically catalysed reaction.[2] Some of the more important variables in an enzyme catalysed transesterification, apart from the alcohol substrate (acyl acceptor), are the acyl donor and its mechanism of equilibrium displacement[3], solvent[4], thermodynamic water activity in the reaction mixture[5], temperature[6], and type of enzyme preparation[7]. Once the alcohol substrate has been established, the application as a whole can, as is often done, be optimized by extensive variation of remaining variables. However, studying the reactions of these catalysts does include some

particular features and cannot be done without considering information from the fields of enzymology and enzyme kinetics.[7,8]

This study compiles some of these features and practical aspects of a biocatalytic study with medium engineering[4a,9] and controlled water activity in order to explore the behaviour of the *Candida antarctica* lipase B (CAB). A model reaction (see Scheme 1) with CAB catalysing the acylation of the Douglas Fir Beetle pheromone[10], 3-methyl-2-cyclohexen-1-ol **1** was developed and run under varying conditions. Enantioselectivity and initial rates of transesterification were monitored.



Scheme 1. The model reaction. Solvent to acyl donor 10:1(vol:vol).

The substrate was selected due to a moderate E value[11], giving reliable determination of the enantiomeric excess of the product at low conversion and clearly identifiable induced variations, and because of its facile chiral separation. The enantiomeric excess of the substrate (ee_s) and the product (ee_p) could be determined during a single chiral GC run. Reintegration procedures or peak width gradient were used for optimizing integration parameters to the different characteristics of the enantiomeric couples. Several different concentrations of the CAB SP 525 lyophilized powder preparation were run with each solvent at each water activity and checked to correlate to initial rates (*i.e.* conversion) calculated from ee_s and ee_p according to Chen *et al.*[8] Calculated E values were constant with enzyme concentration in each point except in the case of 3-pentanone at high water activity (see below).

RESULTS AND DISCUSSION

Activity was monitored through initial rates of transesterification. Two responses towards increased water activity were found. In hexane , benzene, toluene, and 3-pentanone the reaction rates remained at fixed levels with some variation (Fig. 1). Dichloromethane, vinyl acetate, and *t*-amyl alcohol gave significantly higher initial rates of transesterification with water activity, most evident for dichloromethane. This can, to some extent, be ascribed to a lower degree of catalyst aggregation in these solvents.

In general, higher selectivity was associated with low water activity (Fig. 2). Reactions in dichloromethane and *t*-amyl alcohol deviated from this pattern and enantioselectivities remained unaffected by changes in water activity. Most dramatically affected by increasing water activity were reactions in vinyl acetate and hexane with drops in enantioselectivity corresponding to an approximate $\Delta\Delta\Delta G^{\#}$[4c] of 0.7 and 0.6 kcal/mol, respectively.

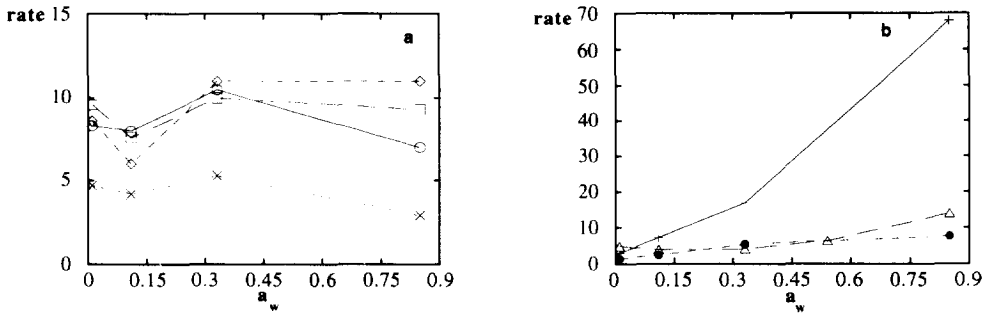


Figure 1a and b. Initial rates of transesterification in nmol/minxmg SP 525 enzyme powder versus water activity in various solvents. 1a: (◊) benzene, (□) toluene, (○) hexane, (x) 3-pentanone. 1b: (+) dichloromethane, (Δ) vinyl acetate, (●) *t*-amyl alcohol. Standard deviation <20%.

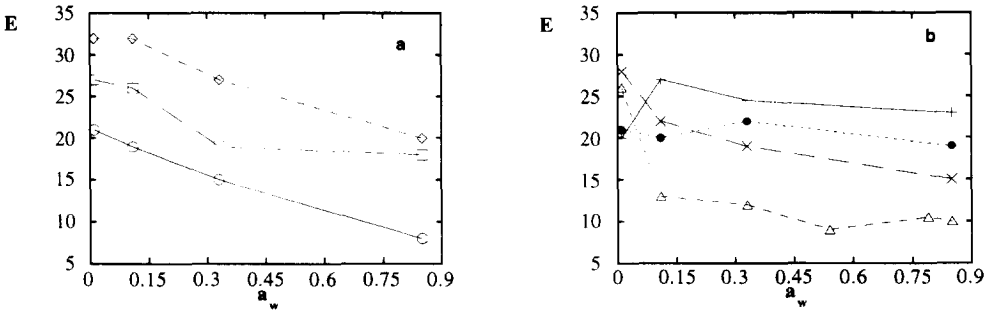


Figure 2a and b. Enantioselectivity (E) as a function of water activity (a_W) in various solvents. 2a: (◊) benzene, (□) toluene, (○) hexane. 2b: (+) dichloromethane, (x) 3-pentanone, (Δ) vinyl acetate, (●) *t*-amyl alcohol. Each point represents an average of 4-8 reactions. Standard deviation lower than 3 except for dichloromethane (a_W=0.85, s.d.=5), hexane (molecular sieves, 6), and 3-pentanone (0.11, 5).

This was initially attributed to an unobserved uncatalysed reaction with the activated ester not occurring under anhydrous conditions. For 3-pentanone such a reaction was found (Fig. 3) and it occured at a rate 3-4% of the total reaction rate. However, in pure vinyl acetate the rate of spontaneous reaction was comparable to or lower than that in *t*-amyl alcohol (<1‰ conversion/h at 45 °C or 2‰ of the total reaction rate, see Fig. 3), which displays constant selectivity with increasing water activity. Further, in 3-pentanone at high water activity apparent enantioselectivity slightly increased with higher enzyme concentration within the set of reactions run at that water activity (see Experimental section), exposing the underlying non-selective spontaneous reaction.

Of the seven solvents examined benzene displayed the most enantioselective reaction (under anhydrous or low water activity conditions). This is in accordance with results found for other lipases where cyclic solvents were suggested for maximum selectivity.[4d] The decreases in selectivity with increasing water activity for toluene and benzene corresponded to $\Delta\Delta\Delta G^{\#}$<0.3. Being a solvent as well as an acyl donor, vinyl acetate is often used

as the medium for applications of this type. Hence, it is of some interest that the use of vinyl acetate as a reaction medium gave significantly lower enantioselectivity than most alternatives here investigated.

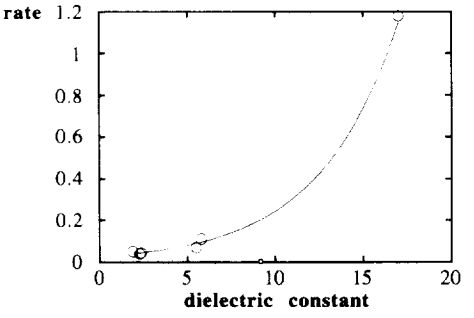


Figure 3. Rate (nmol/min) of spontaneous reaction at high water activity (0.85) versus the dielectric constant of the solvents used. Circles from left to right; hexane, toluene, benzene, vinyl acetate (postulated ε=5.5), *t*-amyl alcohol, and 3-pentanone. The spontaneous reaction rate in dichloromethane (▫) was below the detection limit. A reaction rate of 5 nmol/minxmg enzyme powder in Figure 1 corresponds to 40 nmol/min at lowest enzyme concentration used (4 mg/ml). The curve represents best fit to an equation $y=ke^{mx}$.

Practical Aspects

In a systematic comparative study of a realistic application, a very complex system must often be accepted. It is essential, as has been illustrated here, to control and ensure the validity of the equations used to process the data. Normally this implies an irreversible reaction starting with a perfect racemate avoiding chemical racemization and the uncatalysed formation of product. Assuring a constant apparent value of enantioselectivity with varying enzyme concentration is imperative and a trend in the variation of E is to be regarded a danger signal.

It should be noted that the best fit of experimentally found ee_s and ee_p at varying conversions to the theoretical relation, ee_p=f(ee_s, E), is a more precise way of determining the E value.[12] Here, this method gave results in good agreement with those calculated according to the equations of Chen *et al.*[11]

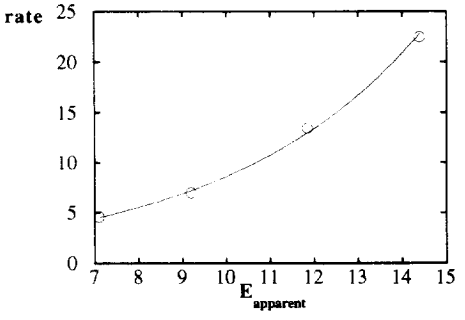


Figure 4. Increasing rate, due to different stirring techniques, and subsequently observed enantioselectivity in hexane at high water activity (0.85). From left to right; orbital shaker (first and second point, each an average of 4 reactions), magnetic stirring (third point, average of 2 reactions), and initial sonication and orbital shaker (last

point, average of 2 reactions). Rate given in nmol/minxmg SP 525 preparation. The phenomenon was not observed in other solvents or at other water activities. The curve represents the best fit to an equation $y=ke^{mx}$.

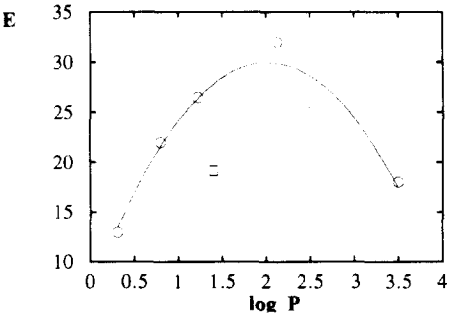


Figure 5. Correlation at water activity 0.11 of selectivity and log *P* of solvents studied. Circles from left to right; vinyl acetate, 3-pentanone, dichloromethane, benzene, toluene, and hexane. Best fit to a second order polynomial function. Deviating *t*-amyl alcohol (▫) is shown. Also ethers are known to deviate.[13]

Effect of Water Activity

Before assuming that increased water activity directly influences enzyme enantioselectivity, alternative explanations were considered. Apart from the uncatalysed reaction observed above with the polar 3-pentanone, aggregation of the catalyst preparation and hydrolysis and thus the amount of acid accumulating, can be escalating problems with increasing water activity. Since these effects slow down the rate of transesterification, a net increase in enzyme activity with water activity is needed for a constant apparent rate of transesterification.

Regarding enantioselectivity, diffusion limitations following aggregation can deminish apparent enzyme enantioselectivity by more severely affecting the fast reacting enantiomer.[14] Experiments in hexane at high water activity with initially sonicated and non-sonicated preparations gave some support for this hypothesis (Fig. 4).

In all cases except two, increasing water activity meant lower enantioselectivity. In dichloromethane, preequilibration with molecular sieves lowered selectivity compared to higher values of water activity. Selectivity seemed unaffected by variation of water activity in *t*-amyl alcohol. No explanation for the dramatic loss of selectivity in vinyl acetate with increasing water activity has been found.

Effect of the Nature of the Solvent

The choice of solvent brings along a "package" of parameters (see below) linked to the physical properties of the compound. Their relative individual importance for activity and selectivity in this kind of reactions is much debated.[4a,b] Here, the enantioselectivity of the powder enzyme preparation showed great dependence on the choice of solvent.

For the enzyme catalysed reaction correlation with neither hydrophobicity nor polarity could be found. However, excluding the "anomalous" *t*-amyl alcohol, selectivity gave the relation to hydrophobicity (log *P*)[15] at the water activity 0.11 showed in Figure 5. That is, a tendency toward optimum selectivity at log $P \approx 2$.

Another characteristic of the solvents is their solubility in water. Except for the particular cases of vinyl acetate and 3-pentanone, water solubility was inversely correlated to loss of enantioselectivity with increasing

water activity. That is, the solvent more soluble in water[16] better retained enantioselectivity with increasing water activity.

CONCLUSION

The effects observed on selectivity with water activity or solvent structure or the combination of the two, amount to $\Delta\Delta\Delta G^{\#}$=0.6 (16), 0.6 (19), and 0.9 kcal/mol (24), respectively (values within brackets are "ΔE_{max}"). In this range of enantioselectivity they make the difference between a possible application and one useless. This illustrates the need of studying and controlling these parameters.

Effects on rate were of diverse character for the different solvents. Under anhydrous conditions, rates differed by no more than a factor two. At high water activity, over one order of magnitude of difference was found with dichloromethane and 3-pentanone. The high rate in dichloromethane is the only reason to apply a higher water activity than 0.11, especially if catalyst preparation is scarce or expensive. In Figure 6, a fast evaluation of the most suitable conditions for the reaction can be found. The upper right part represents the most favourable region. For an acceptable rate and highest possible selectivity, the preparative reaction (see Experimental section) was performed in benzene, all components pre-equilibrated with activated molecular sieves, giving the highest selectivity achieved so far with this pheromone substrate (references 17 and 18: E=2-17).

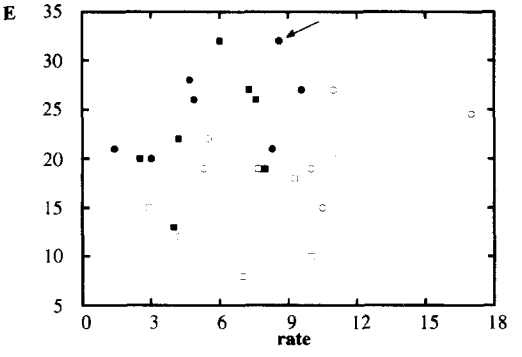


Figure 6. Enantioselectivity vs. rate (nmol/minxmg SP 525 preparation) under all experimental conditions (dichloromethane at a_W 0.85 not shown, E=23, rate=68). Representation: (▫) a_W 0.85, (○)a_W 0.33, (■) a_W 0.11, (●) a_W<0.1.

EXPERIMENTAL

Enzyme

The lipase, *Candida antarctica* (component B), preparation SP 525, containing 40% protein (Lowry assay, rates per mg protein are consequently obtained by multiplying values given by a factor 2.5) with hydrolytic activity typically of 150 000 LU/g, is a product of NOVO-Nordisk A/S, Denmark.

General Procedure

Previous to mixing, all components *i.e.* organic solvents, vinyl acetate, enzyme, and racemic 3-methyl-2-cyclohexen-1-ol (**1**, Aldrich) were separately adjusted to the desired water activity in sealed containers for 2-4 days at 25 °C in the presence of activated molecular sieves (4Å) or with the vapour phase of saturated salt solutions[19] of

known water activity[20]: LiCl (a_w 0.11), $MgCl_2.6H_2O$ (a_w 0.33), $Mg(NO_3)_2{\cdot}6H_2O$ (a_w 0.54), NH_4Cl (a_w 0.79), KCl (a_w 0.85).

At every point of thermodynamic water activity investigated, a set of four reactions with 8, 12, 16, and 20 mg of enzyme preparation, 1.8 ml of solvent, 0.2 ml (1.05 M) vinyl acetate, and 80 µl (0.32 M) of **1** were run simultaneously a fixed reaction time (2-8h, depending on the solvent) giving conversions in the range of 1-9%. Generally, this was done twice. The reactions proceeded in an orbital shaker at 45 °C and were quenched through the removal of the enzyme by filtration.

Higher rates in hexane at high water activity were achieved by initial sonication or vigorous magnetic stirring of reaction mixtures.

Preparative Resolution of 3-Methyl-2-cyclohexen-1-ol

In a two-necked 50 ml flask, compound **1** (1.44 g or 12.8 mmol) was added to a suspension of 300 mg of the SP 525 enzyme preparation in benzene (36 ml) at 45 °C. Tridecane, 378 mg or 0.05 M, was used as an internal standard. All components were preequilibrated with molecular sieves.

Conversion was monitored on a Varian 3500 gas chromatograph with a DB1 column (15m, widebore) and determined to 48.6% after 26 h. The reaction was quenched by filtering off the enzyme and ee_s and ee_p were found to be 75.2 and 85.5%, respectively (GC as described below). According to $ee_s/(ee_p+ee_s)$[12], this gives a conversion of 46.8% and subsequently an enantiomeric ratio of 29[8]. Isolated yields were 69 (0.496 g) and 52% (0.440 g) and optical rotations were $[\alpha]_D$=-4.25° (*c* 0.1, $CHCl_3$) and $[\alpha]_D$=+16.04° (*c* 0.1, $CHCl_3$) of remaining alcohol (**1b**) and product (**2**), respectively. The literature provides absolute configurations according to the following: (*S*)-(-)-**1b** and (*R*)-(+)-**2**.[21]

Gas Chromatography

Instrumentation: Hewlett Packard 5890 Series II and Integrator HP 3396 Series II with Controller 7673. Column: Chrompack CP-cyclodextrin-β–2,3,6–M–19 (50 m, 0.25 mm i.d., 0.25 µm film) at 96 °C with H_2 as carrier gas giving enantiomeric separation factors (α) and resolutions (R) as follows: $\alpha_{remaining\ substrate}$=1.14, $\alpha_{product}$=1.02, R_{rem}=9.06, R_{prod}=2.96. Retention times under these conditions were around 18 minutes for the remaining alcohol enantiomeric couple and around 23 minutes for the product acetate enantiomeric couple.

ACKNOWLEDGEMENTS

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