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Mass Transport Limitations Reduce the Effective Stereospecificity in Enzyme-Catalyzed Kinetic Resolution

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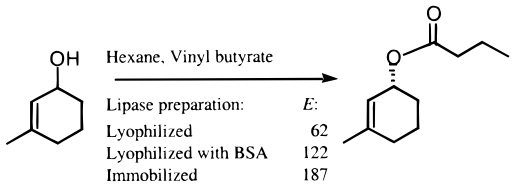
ABSTRACT



The kinetic resolution of seudenol catalyzed by *Candida antarctica* lipase B in hexane was investigated. Large differences in reaction rate and stereospecificity were observed when different enzyme preparations were used. These differences were ascribed to mass transport limitations which reduced both reaction rate and stereospecificity. Lyophilized enzyme preparations were more apt to give this problem than immobilized preparations. Further, low substrate concentrations enhanced the effect. Thus, high alcohol concentrations and enzyme immobilization can be recommended.

Enzyme-catalyzed kinetic resolution is a convenient and widely used method for the preparation of optically active alcohols, acids, and amines.[1] Efforts have been devoted to the engineering of biocatalysts and biocatalytic processes in organic media. Many factors have been found to affect the regio- and stereoselectivity as well as the reaction rate and the enzyme stability in organic solvents. Enzyme formulation is one of these factors. Lyophilization in the presence of various excipients, enzyme solubilization by chemical modification, cross-linking of enzyme crystals, and immobilization on solid supports are some of the procedures that have been shown to improve the enzyme properties.[2] Despite the potential of these procedures, their generality and molecular mechanisms have often remained speculative or unclear. In the present study, we examine the activity and stereoselectivity of several lyophilized or immobilized preparations of a lipase in an organic solvent and report experimental evidence that mass transfer limitations are mainly responsible for the differences found in the enantioselectivity and the reaction rate.

Candida antarctica lipase B (CALB) displays high enantioselectivity toward *sec*-alcohols.[3] This lipase shows high stability and activity in organic media and is available with good purity as a recombinant protein. We compared various CALB preparations as regards their enantioselectivity and reaction rate in catalyzing the transesterification of racemic seudenol (3-methyl-2-cyclohexen-1-ol, a sex pheromone of the Douglas fir beetle). Seudenol was a good candidate because of the easy determination of ee_s and ee_p by means

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of chiral GC and the moderate enantioselectivity reported for the kinetic resolution of this substrate.[4] Thorough medium engineering on the CALB-catalyzed kinetic resolution of seudenol had been performed by Orrenius et al.[4a] The CALB-catalyzed irreversible transesterification reaction in hexane was now studied.[5] Kinetic resolution was carried out with four immobilized (**1**−**3** and **8**) and four nonimmobilized preparations (**4**−**7**).[6] As shown in Table 1, these preparations gave large differences in reaction rate and in stereospecificity. A correlation between the reaction rate and the enantioselectivity was noticed, with the enzyme preparation giving the lower reaction rates generally also showing the lower effective enantioselectivity, E_{eff}.[7,8]

Table 1. Influence of Enzyme Preparations on the Stereospecificity toward Seudenol[a]

entry	CALB preparation[b]	initial rate,[c] mmol min^{-1} g^{-1}	E_{eff} ± SD[d]
1	Chirazyme L2, c-f, lyo	0.17	98 ± 2
2	Novozym 435	0.45	179 ± 9
3	Chirazyme L2, c-f, C3, lyo	0.53	187 ± 7
4	free CALB, lyo	0.013	62 ± 5
5	6.5% free CALB, lyo with BSA	0.13	96 ± 8
6	1.2% free CALB, lyo with BSA	0.11	122 ± 12
7	6.5% free CALB, lyo with inhibited CALB	0.074	93 ± 5
8	0.9% CALB on Accurel	33	187 ± 12

[a] Reaction conditions: seudenol (0.2 M, 0.119 mL), vinyl butyrate (0.4 M, 0.253 mL), hexane (4.6 mL), and lipase. [b] See footnote 6. [c] Entries **4**−**8** per g of lipase; entries **1**−**3** per g of lipase and carrier. [d] SD = standard deviation.

These findings can be related to mass transport problems. In organic solvents, enzymatic catalysis is carried out under heterogeneous conditions due to the poor solubility of most enzymes in organic media. These conditions restrict the mobility of both the enzyme and the solutes, which can lead to mass transfer limitations. Since the concentration of the fast reacting enantiomer will be depleted faster that of the slow reacting one, the effective enantioselectivity may be reduced.[9]

Our experiments were performed at 500 rpm, and increased stirring changed neither the rate nor the enantioselectivity. Thus, we ruled out external diffusion limitation as the factor influencing the enantioselectivity.

On the other hand, internal diffusion restrictions are likely to occur in porous particles such as aggregates and immobilized enzymes. A mathematical description of the diffusion limitations for enzymes obeying the Michaelis−Menten kinetics has been established.[10] The extent of mass transfer control can be expressed by the effectiveness factor η, which is the ratio between the observed rate of reaction and the hypothetical rate in the absence of mass transfer. The effectiveness factor η is a function of the Thiele modulus ϕ_R and the dimensionless substrate concentration β.[11]

The lyophilized preparation **4** caused the slowest reaction rate and showed the lowest enantioselectivity. Moreover, this preparation aggregated and stuck to the glass wall of the vessel. This lyophilized preparation contained 60% w/w active protein according to active-site titration.[12] Since high enzyme concentrations favor mass transport problems, we decreased the local enzyme concentration by adding an excipient: preparation **4** was dissolved in a buffer solution containing bovine serum albumin (BSA) and relyophilized.[13] The two lyophilized preparations so obtained (**5**−**6**) were more enantioselective and caused higher reaction rates than the original preparation **4** (Table 1). This led us to believe that mass transport limitations could indeed be present in **4**. However, it was not clear whether the effects observed with the BSA preparations were due to other factors than diffusion limitations.

Two strategies were applied to confirm the presence of mass transport limitations. At first the lyophilized preparation **4** was lyophilized as previously described for the BSA preparations, but using an inhibited CALB (**4**) as an excipient instead of BSA.[14] The similarity of preparation **7** with BSA preparation **6** proved that the BSA in **6** was not directly responsible for the increase in the E value and the reaction rate. Instead, BSA acted mainly on the local enzyme concentration in the aggregate. Second, preparation **4** was immobilized by adsorption onto Accurel EP100 at pH 7.5. The preparation obtained, **8**, was 3-fold more enantioselective

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(5) The CALB preparations were equilibrated against a saturated aqueous LiCl solution for 2 days (a_w = 0.1). In a sealed vial, (±)-3-methyl-2-cyclohexen-1-ol (1−2 mmol) was added to the CALB preparation suspended in hexane (previously dried over molecular sieves). The mixture was stirred at 500 rpm in a thermostated multistirrer at 23 °C for 30 min. Vinyl butyrate (2 equiv, 2−4 mmol) was added to the solution, and samples were taken regularly. The enantiomeric excesses, ee_s and ee_p, were monitored by chiral GC (Chrompack CP Chiralsil-dex CB 25 m × 0.32 mm or a J&W scientific CycloSil-B 30 m × 0.32 mm, FID detector). The enantiomeric ratio values, E, were based on four−six measurements at different conversions, all lower than 50%. E was calculated using the formula $E = \ln[(1 - ee_s)/(1 + ee_s/ee_p)]/\ln[(1 + ee_s)/(1 + ee_s/ee_p)]$ (Rakels, J. L. L.; Straathof, A. J. J.; Heijnen, J. J. *Enzyme Microb. Technol.* **1993**, *15*, 1051) and were computed with the program Simfit (van Tol, J. A. B.; Jongejan, J. A.; Geerlof, A.; Duine, J. A. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 255).

(6) Chirazyme L2, c-f, lyo (**1**), Novozym 435 (**2**), and Chirazyme L2, c-f, C3, lyo (**3**) are immobilized CALB preparations. The preparation (**2**) was a gift from Novo Nordisk and the others (**1**,**3**) from Boehringer Mannheim. The preparations **4**−**8** were all prepared from a highly purified lyophilized CALB supplied by Novo Nordisk.

(7) The enantioselectivity in an enzymatic kinetic resolution process is expressed by the enantiomeric ratio, E. This is the ratio between the specificity constants (k_{cat}/K_M) of the enzyme for the competing R and S enantiomers; $E = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S$.

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(10) Lee, Y. Y.; Tsao, G. T. *J. Food Sci.* **1974**, *39*, 667.

(11) For a spherical geometry, $\eta = f(\phi_R, \beta)$. The Thiele modulus, ϕ_R, is defined as $\phi_R = R \times \sqrt{V_m/(D_{eff} \times K_M)}$ and the dimensionless substrate concentration, β, as $\beta = K_M/S$ where S is the bulk-phase substrate, D_{eff} is the effective diffusivity, R is the radius of the support, K_M is the Michaelis constant, and V_m is the maximum reaction rate in the Michaelis−Menten equation.

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(13) Lyophilization: CALB (1.7 mg) and BSA (26 or 156 mg) were dissolved in 3 mL of 5 mM TRIS pH 7.5 and lyophilized in a Speedvac.

and 1000-fold more active toward seudenol than the original preparation **4**. Furthermore, the enantioselectivity of **8** reached a level similar to that of the most enantioselective preparations. Thus, we concluded that mass transport limitations were indeed the main cause of the difference in enantioselectivity between the lyophilized enzyme preparations.

Concerning the immobilized preparations, preparation **1** displayed a lower reaction rate and enantioselectivity than the other immobilized preparations. This could also be due to mass transport limitations. The diameters of the carrier and the enzyme loading are parameters that influence the diffusion limitations of an immobilized enzyme.[15] These two parameters had been used to detect and study diffusion limitations.[15,16] In our case, the diameter and loading of the immobilized preparations **1**−**3** had been fixed by the manufacturer and could not easily be changed. On the other hand, the substrate concentration is a parameter that can easily be changed.

An increase of the substrate concentration may prevent the depletion of substrate at the center of the carrier. This increase has been shown to reduce the internal diffusion limitations of a single substrate. Substrate concentrations 100-fold higher than the K_M value have been recommended.[15] Furthermore, substrates often have high apparent K_M values in organic media.[17] Thus, mass transfer problems are even more likely to occur in these media than in water. If internal diffusion limitations are present, increasing the substrate concentration could increase the apparent enantioselectivity or even restore the true selectivity. The effect of the substrate concentration on the effective enantioselectivity has been modeled and simulated for a continuously operated fixed bed reactor by Indlekofer et al.[18] We tested the effect of the substrate concentration on the E_{eff} of two immobilized preparations **1** and **2**. They displayed E_{eff} values of 98 and 186, respectively, under 0.2 M seudenol concentration. Further increase of the substrate concentration with preparation **2** did not lead to any significant change in the *E* value (Table 2).

Table 2. Influence of Substrate Concentration on the Enantioselectivity toward Seudenol

CALB preparation	seudenol, M	initial rate,[a] mmol min^{-1} g^{-1}	$E_{eff} \pm$ SD[c]
Chirazyme L2, c-f, lyo	0.02	0.028	67 ± 2
Chirazyme L2, c-f, lyo	0.2	0.17	98 ± 2
Chirazyme L2, c-f, lyo	0.8	0.45	141 ± 4
Chirazyme L2, c-f, lyo	1.6	0.52	154 ± 1
Novozym 435	0.02	0.16	119 ± 8
Novozym 435	0.02[b]	0.14	124 ± 5
Novozym 435	0.2	0.45	200 ± 7
Novozym 435	0.8	0.53	199 ± 5
Novozym 435	1.6	0.78	176 ± 7

[a] Per gram of carrier. [b] 80 equiv of acyl donor instead of 2. [c] SD = standard deviation.

On the other hand, the E_{eff} of preparation **1** increased dramatically when the substrate concentration was increased. Furthermore, at high substrate concentration all immobilized preparations reached a similar *E* value, which we believe represented the true CALB enantioselectivity toward seudenol. At the lowest substrate concentration (0.02 M) the two immobilized preparations **1** and **2** displayed their lower E_{eff} due to enhanced mass transport limitations. We concluded that mass transport limitations were the main cause of the differences in enantioselectivity between the immobilized preparations tested.

We also desymmetrized *meso*-2,3-butanediol in methyl *tert*-butyl ether.[19] If diffusion limitations are alone responsible for the difference in *E*, the selectivity should be the same for all of the preparations tested.[20] The *E* values presented in Table 3 were calculated at conversions at which less than 1% diester had been formed. All of the preparations displayed similar selectivity toward *meso*-2,3-butanediol which supports the above evidence that mass transport limitations are responsible for the differences in enantioselectivity between the preparations tested.

Table 3. Desymmetrization of *meso*-2,3-Butanediol

CALB preparation	diol, M	$E_{eff} \pm$ SD[a]
Chirazyme L2, c-f, lyo	0.05	11.3 ± 1.2
Chirazyme L2, c-f, lyo	0.2	10.4 ± 0.9
Novozym 435	0.05	9.0 ± 0.2
Novozym 435	0.2	9.5 ± 0.4
Chirazyme L2, c-f, C3, lyo	0.05	12.8 ± 0.3
Chirazyme L2, c-f, C3, lyo	0.2	13.0 ± 0.5
free CALB, Lyo	0.05	10.0 ± 0.8
6.5% free CALB, lyo with BSA	0.05	11.4 ± 1.0

[a] SD = standard deviation.

In organic solvents, enzymatic activity and enantioselectivity have been shown to be dependent on the history of the enzyme.[21] Klibanov suggested that diffusion limitations

(14) CALB **4** (26 mg) was dissolved in 2.4 mL of 5 mM TRIS buffer pH 7.5. Methyl *p*-nitrophenyl hexylphosphonate (0.1 mL, stock solution 10 mM in CH_3CN) was added, and the reaction was monitored at 400 nm. After complete irreversible inhibition, the protein was dialyzed and lyophilized.

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(19) The CALB preparations were equilibrated against a saturated aqueous LiCl solution for 2 days (a_w = 0.1). In a sealed vial, *meso*-2,3-butanediol (1−2 mmol) was added to the CALB preparation suspended in methyl *tert*-butyl ether (previously dried over molecular sieves). The mixture was stirred at 500 rpm in a thermostated multistirrer at 23 °C for 30 min. Vinyl butyrate (2 equiv, 2−4 mmol) was added to the solution, and samples were taken regularly. ee_p was monitored by chiral GC (J&W scientific CycloSil-B 30 m × 0.32 mm, FID). The *E* values were based on four−six measurements.

(20) *meso*-2,3-Butanediol bears both the *R* and the *S* hydroxyl group on the same molecule. Thus, the local concentrations of the *R* and *S* stereocenters will remain identical throughout the support and only the substrate concentration will be reduced if mass transfer limitations are present.

are not responsible, as widely claimed, for the differences in rates in water and in organic solvents.[22] Indeed, many causes for the alterations, between preparations, in activity and selectivity in organic solvents have been identified.

Concerning mass transfer, some researchers did not find any mass transport limitations with the protease subtilisin under their experimental conditions.[23] On the other hand, mass transfer limitations with immobilized preparations of the protease α-chymotrypsin have been reported in organic media.[16] The differences in reaction rates and enantioselectivity reported in this Letter were explained in terms of mass transport problems. Thus, the differences observed in our study should not be related to conformational differences between enzyme preparations in hexane.

The *E* values presented were higher than the ones previously reported for the kinetic resolution of seudenol catalyzed by CALB.[4a] The authors of that paper used a lyophilized preparation containing 40% protein, and they suggested possible diffusion limitation problems in their system. The higher *E* values reported here would support their hypothesis although the model reactions used were not identical.

Our experimental results provide evidence that diffusional effects are important in the enzyme-catalyzed kinetic resolution in organic media. Mass transport limitations are detrimental to enantioselectivity and reaction rate and should thus be avoided in kinetic resolution. Overlooking these can easily lead to a decrease in the enantiomeric excess and the reaction rate. Diffusion limitations can be minimized by the use of immobilized preparations at high substrate concentrations. For instance, screening for suitable biocatalysts at low substrate concentrations to save precious substrate is more likely to result in faulty choices than the use of high concentrations. If the enzyme is immobilized "in house", low enzyme loading and small particle size will help to overcome mass transport limitations.

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