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Substrate sorption into the polymer matrix of Novozym 435® and its effect on the enantiomeric ratio determination

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Abstract—In the enantioselective esterification of 4-methyloctanoic acid with ethanol by immobilised *Candida antarctica* lipase B (Novozym 435®), the enantiomeric excesses determined during the course of the reaction deviated strongly from the theoretical values, leading to unacceptably large confidence intervals for the enantiomeric ratio (*E* value). This observation was in contrast to our previous findings for transesterification and hydrolysis reactions with this enzyme. Herein, the three reactions are compared; the anomalous results in the esterification reaction appear to be caused by adsorption of 4-methyloctanoic acid inside the enzyme beads. We found that on average 1.19 g of 4-methyloctanoic acid was incorporated per g of Novozym 435®. If the concentration of this substrate was adjusted accordingly in the calculations, the resulting *E* values showed acceptable confidence intervals. In previous research on transesterification reactions in excess apolar solvent (comparable affinity for the beads), sorption does not play an important role because only small amounts of substrate were lost. For hydrolysis reactions, sorption takes place but the acid is released from the beads upon titration and no effect on the *E* value is found. However, for esterification reactions, sorption should not be neglected since there is no driving force to release the acid from the beads.

1. Introduction

Nowadays, lipases are well-accepted catalysts in organic chemistry, both in aqueous and non-aqueous media. Working with these enzymes in organic solvents has several potential advantages, e.g. the possibility to shift the thermodynamic equilibrium to favour esterification over hydrolysis.[1–5] In this way, the kinetic resolution of acids or alcohols can be performed by lipase-mediated esterification in organic solvents.[6–10]

To evaluate a kinetic resolution in terms of enantioselectivity, the enantiomeric ratio (*E*) has been defined. This parameter corresponds to the ratio of the specificity coefficients, V_{max}/K_M, of the individual substrate enantiomers. Several methods exist for the determination of *E*.[11–16]

Recently, we have studied the kinetic resolution of some branched chain fatty acids and their esters.[17–19] Novozym 435® (immobilised *Candida antarctica* lipase B, CALB) showed the best results ($E=23$) in the transesterification reaction of 4-methyloctanoic acid methyl ester to its butyl ester.[17] Also this enzyme was successfully applied in hydrolysis reactions.[19] The same enzyme is now used for the esterification of 4-methyloctanoic acid with ethanol in a solventless system (see Scheme 1).[19] The effect of substrate sorption is investigated in detail and the results are compared to those obtained for hydrolysis and transesterification.

2. Results and discussion

2.1. Determination of the enantiomeric ratio in the esterification of 4-methyloctanoic acid

Esterification of 4-methyloctanoic acid was performed using a range of concentrations varying from 2:1 up to

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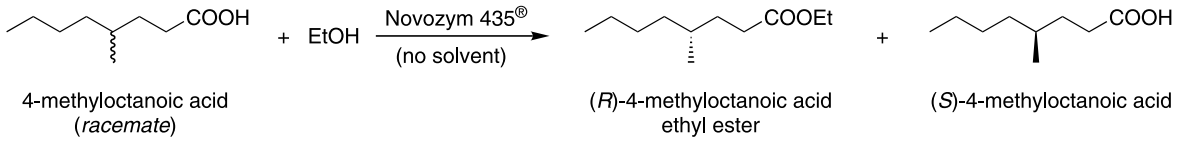

Scheme 1.

1:20 mol fatty acid to mol ethanol (see Table 2 for the exact range). Novozym 435® was able to catalyse this reaction efficiently in the absence of solvent and in the presence of a large amount of polar substrate (ethanol, log $P=-0.24$).[19] Except for the 2:1 ratio, all the reactions reached approximately 80% conversion of 4-methyloctanoic acid after 100 h. The enantiomeric excess of the substrate was determined from the GC-data and plotted versus the measured conversion.

The enantiomeric ratio, the equilibrium constant and their confidence intervals were calculated with a computer program[13] that uses the Chen equation for competitive Michaelis Menten kinetics for equilibrium reactions.[12] Figure 1 shows the calculated E values and their confidence intervals for all fatty acid to ethanol ratios studied. In most of the cases, there was poor agreement between the Chen-model and the experimental data, resulting in large confidence intervals for the estimated E values. For the ratio 1:8, a confidence interval is shown from 0 to 140 because in this case the Chen equation could not be used to fit the data. The poor fits could not be attributed to enzyme inactivation, since the reuse of CALB showed the same enzyme activity (results not shown).

It may be that a part of the substrate is not readily available for the reaction, for instance because of swelling of the enzyme support in the presence of organic solvent. If this is the case, this leads to an overestimation of the conversion. The liquid inside the beads can act as a 'dead' volume if diffusion is slow compared to the reaction time. However, an analysis of the characteristic times for the processes that take place revealed that slow diffusion cannot explain the effects on the E-value in Figure 1 (results not shown). This means that there must be another reason for our anomalous results. Physical adsorption of the substrate to the polymer beads would be a possibility. We consulted the manufacturer of the enzyme carrier (Bayer AG) and they were kind enough to confirm our suspicion that sorption of the substrate to the carrier can take place (Rudolf Wagner, personal communication). This is also true for other hydrophobic molecules. In these cases, part of the liquid is not available for reaction. Therefore, we decided to study the swelling behaviour in more detail and quantify these effects.

2.2. Swelling of enzyme beads

Swelling of polymer materials in the presence of solvents is a common and widely known phenomenon.[20–25] Since in our case, swelling may explain the poor fits obtained, an attempt was made to measure the swelling quantitatively. Different techniques were used to measure the volume increase that results from the diffusion of ester or acid into the macroporous beads of the polymer on which *C. antarctica* lipase B is immobilised.

In order to monitor changes in the structure of the enzyme beads or increase in volume, Novozym 435® was equilibrated with pure 4-methyloctanoic acid or its ethyl ester. The hydrophobic material was studied under a microscope. Because of sorption in the pores, the hydrophobic beads changed from opaque into a larger translucent structure (Fig. 2). At the same time, the mean diameter increased. A reliable mean value of the diameter was obtained by taking the average of approximately 300 beads of both enzyme beads equilibrated with 4-methyloctanoic acid or its ethyl ester, and 'dry' Novozym 435® beads. In Figure 3, the distribution of the diameter was plotted for the three different cases. For the wetted enzyme beads, the distribution shifted towards the right, which implies an increase in diameter. The Sauter mean diameter (d_{32}) of the beads wetted in 4-methyloctanoic acid and in 4-methyloctanoic acid ethyl ester does not differ significantly since the confidence intervals overlap (Table 1). Assuming spherical

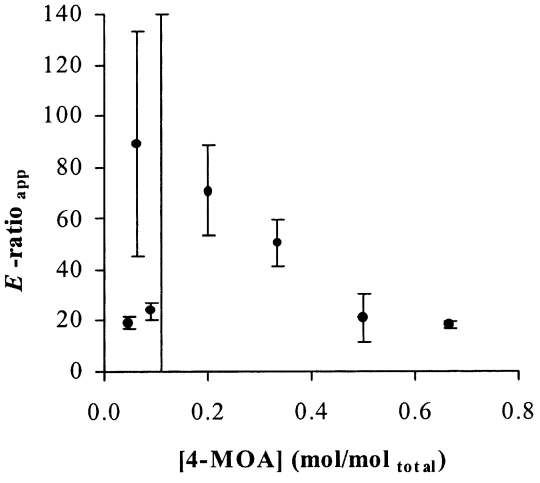

Figure 1. Esterification of 4-methyloctanoic acid with ethanol, catalysed by Novozym 435® at different ratios varying from 2:1 up to 1:20 (mol fatty acid: mol ethanol). The estimated E values and their confidence intervals were calculated using the equation of Chen et al.[11,12] in the program SIMFIT by Jongejan.[13]

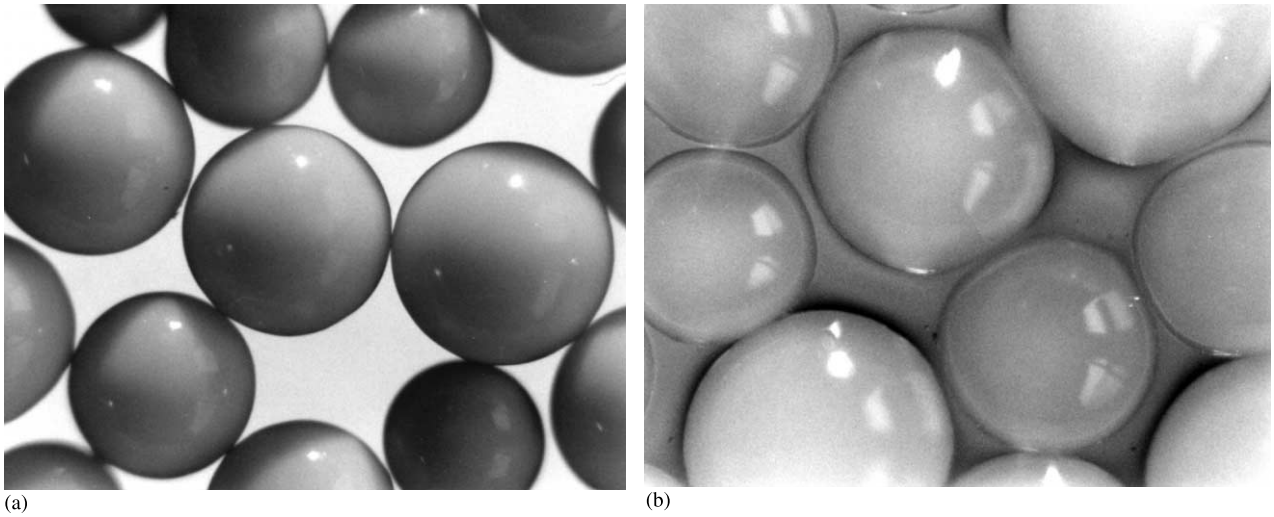

Figure 2. (a) 'dry' Novozym 435®, (b) swollen Novozym 435® equilibrated in 4-methyloctanoic acid.

beads the volume of both the swollen and 'dry' beads could also be calculated. From these calculations, it can be concluded that the volume increased with 67±17 nL. Assuming this increase is caused by sorption of acid and no contraction takes place, the volume increase (175–250%) is caused by 7.2±2.6 mmol of fatty acid per gram of enzyme beads.

Table 1. Sauter mean diameter (d_{32}) and volume of wetted and 'dry' Novozym 435®

	d_{32} (μm)	Mean volume (nL)
Dry beads	$5.07\times10^2\pm0.21\times10^2$	58.5±4.0
Beads wetted in acid	$6.50\times10^2\pm0.28\times10^2$	125.3±12.4
Beads wetted in ester	$6.15\times10^2\pm0.16\times10^2$	111.4±5.9

To determine the time needed for absorption/swelling, the swelling of five different beads was taped on video. For each bead, the relative volume is plotted against time in Figure 4. Within 2 min, the volume reached its maximum.

Since our esterification reactions took place on a time scale of more than 100 h, it can be concluded that at the initial stage of the esterification reaction the enzymes were saturated with 4-methyloctanoic acid. Although Figure 4 shows a wide range of maximal relative volumes, the absolute volume increase, $\Delta V=$ 72±4.6 nL, equalled the value calculated from Table 2. The large confidence interval is a consequence of the fact that only five beads were investigated.

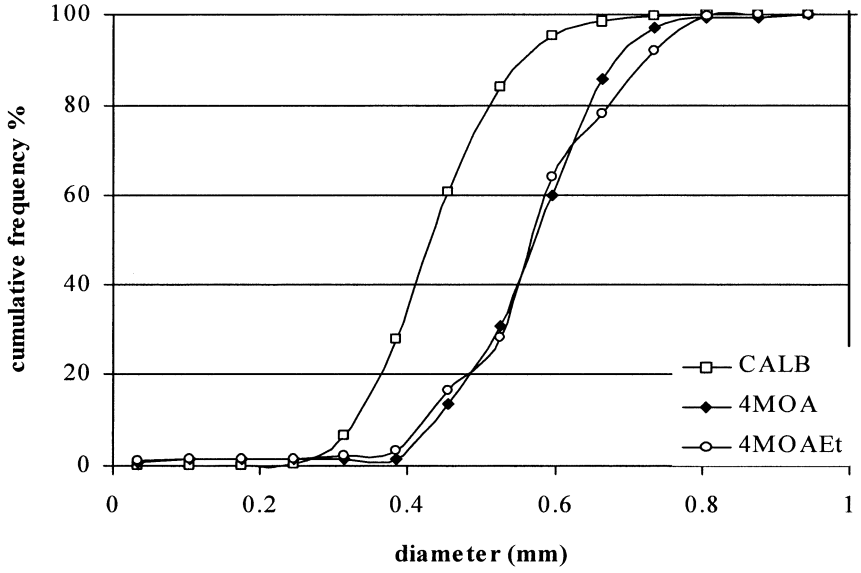

Figure 3. Frequency distribution of the calculated diameters from 'dry' CALB and CALB wetted either in 4-methyloctanoic acid or in 4-methyloctanoic acid ethyl ester.

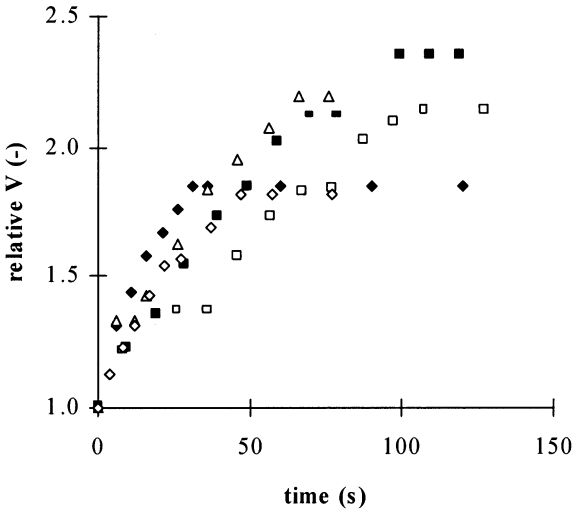

Figure 4. Swelling of Novozym 435® due to the sorption of 4-methyloctanoic acid. The swelling was recorded for five single beads (△, ■, □, ◆, ◇).

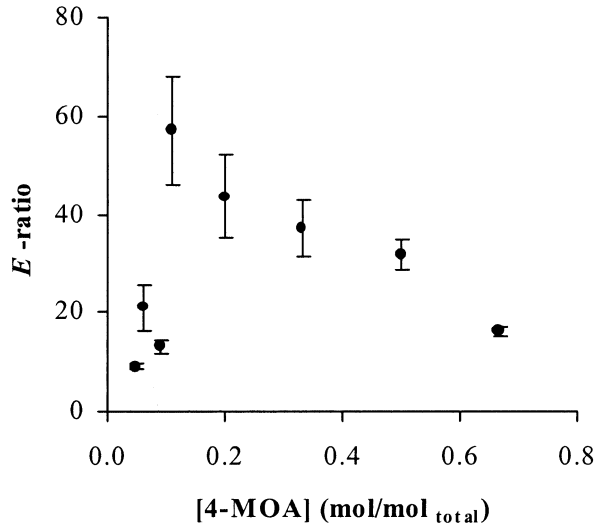

Figure 5. Esterification of 4-methyloctanoic acid with ethanol, catalysed by Novozym 435® at different ratios varying from 2:1 up to 1:20 (mol fatty acid:mol ethanol). The estimated E values and their confidence intervals were calculated using the equation of Chen et al.[11,12] in the program SIMFIT by Jongejan.[13] Note the difference in scale in Figure 1 and Figure 5.

Table 2. Ratios of substrates used in the experiments

Ratio A:E[a]	4-MOA		EtOH	
(mol:mol)	(g)	(mmol)	(g)	(mmol)
2:1	1.743	11.01	0.265	5.75
1:1	1.580	9.98	0.475	10.31
1:2	1.263	7.98	0.749	16.26
1:4	0.927	5.86	1.086	23.57
1:8	0.607	3.84	1.430	31.04
1:10	0.517	3.27	1.508	32.73
1:15	0.377	2.38	1.689	36.66
1:20	0.295	1.86	1.701	36.92

[a] A = 4-methyloctanoic acid, E = ethanol.

2.3. Correction of the enantiomeric ratio

As described previously, during esterification of 4-methyloctanoic acid, a part of the substrate diffuses into the enzyme beads, causing swelling of these particles. The reaction under study is an equilibrium reaction, therefore, the driving force for the release of this absorbed substrate from the bead is considerably smaller (if even present) than would be the case for reactions that go to completion. This part of the substrate is assumed not to take part in the reaction. Therefore, the amount of substrate sorbed by Novozym 435® beads must be subtracted from the initial substrate concentration so that the 'true' conversion can be calculated. The average value of 7.2 mmol per g lipase was used to correct the conversion for sorption and to estimate the actual E values (Fig. 5). The data could now be described adequately by the Chen equation. Figure 5 shows the E value as a function of the 4-methyloctanoic acid concentration with relatively small confidence intervals. This means that the substrate sorption is playing a crucial role here; product sorption, although possible, does not play a significant role. Apparently, the affinity of the carrier for the substrate is larger than for the product.

Figure 5 shows that with decreasing 4-methyloctanoic acid concentrations, the enantiomeric ratio increases up to an optimum of $E = 57$ at 0.11 mol/mol. When the 4-methyloctanoic acid concentration is further decreased (ratios: 1:10, 1:15 and 1:20 (mol:mol)) the enantiomeric ratio drops. These results suggest that the conformational stability of the *C. antarctica* lipase is dependent on the protein-solvent interaction and the enzyme structure.[26–29] It may be that at relatively high 4-methyloctanoic acid concentrations the enzyme is in a more hydrophobic environment leading to a more rigid enzyme, thus enhancing the enantioselectivity. The reason why the enantioselectivity decreases again if the 4-methyloctanoic acid concentration is lowered further is not known. Berglund et al.[30] observed a drop in E value upon decreasing the concentration of 2-methyldecanoic acid in the *Candida rugosa* lipase-catalysed esterification of 1-heptanol, which was caused by the fact that heptanol influences V^S_{max} and V^R_{max} in different ways. So, in our system, two effects (conformational versus kinetic) may be operating simultaneously, which may be the reason for the observed optimum in E value.

2.4. Comparison with transesterification and hydrolysis

Previously, we presented an enantiomeric ratio of 23 in the transesterification of 4-methyloctanoic acid methyl ester to its butyl ester,[17] also catalysed by Novozym 435c. In this reaction, the mixture for transesterification consisted of 500 mM *n*-butanol and 100 mM 4-methyloctanoic acid methyl ester in an excess of octane (1:10).

A comparison of the estimated E for the plot of the measured ee_s versus c and the measured ee_s versus ee_p showed an E value of 23 for both plots. Based on these findings, it is expected that substrate sorption did not play an important role in this case. Both octane and 4-methyloctanoic acid are expected to sorb equally well (personal communication, Bayer AG). Also because the octane is in large excess, the loss of substrate is so small that it apparently does not influence the results.

In hydrolysis reactions, the fatty acid production is commonly measured using in-line titration. Although 4-methyloctanoic acid ethyl ester sorbs into Novozym 435®, the acid produced is extracted to the water phase. In the bulk solution, the acid will be titrated with KOH, driving the reaction towards completion. This was confirmed by a separate experiment in which swollen beads (containing 4-methyloctanoic acid) were titrated. The total amount of acid that sorbed into the beads was completely removed by titration. In cases like this, the enantiomeric excess of the product (acid) should be used to determine the enantiomeric ratio.[19]

3. Conclusions

For the Novozym 435® catalysed esterification of 4-methyloctanoic acid at different substrate concentrations, we found that we could not fit the Chen equation for equilibrium reactions to our data in a reliable way. This was caused by the fact that part of the substrate sorbed into the enzyme beads. Microscopic techniques showed that the amount of 4-methyloctanoic acid sorbed into Novozym 435® approximated 7.2 mmol per g lipase. Since this amount does not take part in the esterification, a correction must be made to calculate the actual conversion. This resulted in reliable plots yielding E values with an acceptable confidence interval. The highest E value (57) was found for an acid/ethanol ratio of 1:8 (mol:mol). The effect of substrate sorption was only found important for esterification reactions; the transesterification and hydrolysis reactions that were investigated previously were not affected by sorption effects.

Novozym 435® (immobilised *C. antarctica* lipase B) is one of the enzymes that are most often used in organic chemistry.[31] However, for other enzymes that are immobilised on organic polymers, sorption of the substrate or product should also be taken into account when performing kinetic measurements in solventless reaction systems.

4. Experimental

4.1. Chemicals

4-Methyloctanoic acid (4-MOA) was purchased from Oxford Chemicals. Glacial acetic acid, toluene and octane were obtained from Acros. Tetradecane was purchased from Aldrich, ethanol was obtained from Merck and dichlorodimethylsilane was supplied by Sigma. All solvents were of analytical quality.

4.2. Enzyme

Candida antarctica lipase B (Novozym 435®) is a product of NOVO Nordisk A/S Denmark. The support material consisted of a divinylbenzene-crosslinked, hydrophobic macroporous polymer based on methyl and butyl methacrylic esters. The lipase is adsorbed onto the surface of the support material by hydrophobic interaction.

4.3. Apparatus

Microscope: Olympus automatic exposure photo micrographic system model PM-10AK, Olympus C35DA-2 photo camera (4×10), Magic Imaging MTI series 68. *Video*: Olympus BH2 microscope, Sony model DXC-151AP camera.

4.4. Esterification of 4-methyloctanoic acid

Esterification was carried out in 4 mL screw-cap vials placed in a New Brunswick Scientific Innova™ incubator at 35°C and 350 rpm for up to 100 h. The vials contained the substrates 4-methyloctanoic acid and ethanol in different molar ratios, as well as tetradecane (internal standard, 0.05 g) and immobilised *C. antarctica* lipase B (20–30 mg of CALB). The total volume was approximately 2 mL. During the course of the reaction 20 μL samples were taken and diluted to 1 mL with octane, containing 0.5% (v/v) acetic acid. The amounts used are given in Table 2.

4.5. GC analysis

Aliquots of 0.1 μL were analysed on a gas chromatograph using a GC-8000 series Fisons Instruments (8160) with a MFC 800 control unit and an A200S CE Instruments autosampler. The GC was equipped with a chiral γ-DEX™ 120 WCOT SUPELCO capillary column (ID: 30m×0.25 mm, film: 0.25 μm).[32] Hydrogen was used as the carrier gas (70 kPa). A temperature program was used to separate both the enantiomers of 4-methyloctanoic acid and 4-methyloctanoic acid ethyl ester ($T_1 =$ 75°C, $t_1 = 53$ min, $r_1 = 5$°C/min, $T_2 = 115$°C, $t_2 = 33$ min). The peaks were detected by an FID (EL 980 Fisons Instrument) set at 200°C. The split temperature was 225°C. The GC was connected to the computer program Xchrom/Windows 4.0 version 2.11b for Windows NT (ThIS Labsystems) for online data acquisition. Tetradecane was used as an internal standard in all measurements. The retention times for 4-methyloctanoic acid ethyl ester were 49.25 and 49.79 min [$\alpha = 1.05$], for 4-methyloctanoic acid 82.77 and 84.10 min [$\alpha = 1.02$]). The liner of the GC was replaced by a new silylated liner every week, since injection of fatty acids leads to a desilylation reaction which gives rise to the adsorption of the fatty acids to the glass. Liners were silylated in a solution of dichlorodimethylsilane (5%) in toluene, rinsed with methanol and dried. A piece of silylated glass wool was placed into the liner to protect the main column from contamination.

4.6. Characterisation of CALB beads

4.6.1. Weight. The mean weight of one enzyme bead was determined by weighing 5 series of 10 beads and taking the average. One bead of Novozym 435® (*C. antarctica* lipase B, CALB) did have an average weight of $5.4\times10^{-2}\pm1.0\times10^{-2}$ mg and so 1 g of Novozym 435® thus contained 18500±2900 beads.

4.6.2. Diameter and volume. Approximately 300 'dry' CALB beads, 300 CALB beads soaked in 4-methyloctanoic acid and 300 CALB beads soaked in 4-methyloctanoic acid ethyl ester were scanned. For each bead the diameter was determined using the program RESULTS from Applied Imaging, version 5.1, 1991.

The Sauter mean diameter was calculated using:

$$d_{32}=\frac{\sum n_i\cdot d_i^3}{\sum n_i\cdot d_i^2} \quad (1)$$

The mean volume was calculated using:

$$V=\frac{1}{n}\sum_{k=1}^{n}\frac{4}{3}\pi\cdot r_k^3 \quad (2)$$

with $n=390$ (dry CALB beads), 305 (CALB beads saturated with 4-methyloctanoic acid) and 381 (CALB beads saturated with 4-methyloctanoic acid ethyl ester).

4.6.3. Rate of swelling. The swelling of enzyme beads was recorded on videotape. To one bead, viewed under the microscope, a drop of 4-methyloctanoic acid was added. The swelling was recorded in time. The volume was calculated in time using Eq. (2).

4.7. Determination of *E* values

For the esterification of 4-methyloctanoic acid with ethanol, the enantiomeric ratio was determined solely using enantiomeric excess data of the substrate and the extent of conversion. This was necessary because of the poor separation of the 4-methyloctanoic acid ethyl ester enantiomers on the GC. Plotting the ee_s versus the conversion in the computer program SIMFIT[13] gave the enantiomeric ratio.

To determine the peak areas of the 4-methyloctanoic acid enantiomers at t_0, a sample was taken from the mixture in the absence of lipase. In case of substrate sorption, the initial amount of 4-methyloctanoic acid (mol/g) was different from the one determined by GC at t_0. Therefore, the conversions that were calculated from the GC data at time t and t_0 were corrected for 4-methyloctanoic acid (mol/g) sorbed into the enzyme beads. Plotting the ee_s versus the conversion in the SIMFIT program[13] gave new E values.

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