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The utility of cyclodextrins in lipase-catalyzed transesterification in organic solvents: enhanced reaction rate and enantioselectivity †

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The use of enzymes as valuable catalysts in organic solvents has been well documented. However, some of their features limit their application in organic synthesis, especially the frequently lower enzyme activity under nonaqueous conditions, which constitutes a major drawback in the application of enzymes in organic solvents. In addition, many enzymatic reactions are subject to substrate or product inhibition, leading to a decrease in the reaction rate and enantioselectivity. To overcome these drawbacks and to make enzymes more appealing to organic chemists, we demonstrate the use of cyclodextrins as regulators for the *Pseudomonas cepacia* lipase (PSL) and macrocyclic additives to enhance the reaction rate and enantioselectivity *E* in lipase-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol in organic solvents. Both reaction rate and enantioselectivity were significantly enhanced by several orders of magnitude when using co-lyophilized lipase in the presence of cyclodextrins. The effect of cyclodextrin derivatives as well as solvents on the improvement of the reaction parameters has been studied. The observed enhancement was tentatively interpreted in terms of their ability to give a certain flexibility to the enzyme and to form a host–guest complex, thus avoiding product inhibition and leading to enhancement of the reaction rate and enantioselectivity. The effect of cyclodextrin additives on the enzyme morphology has been studied using scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) of the co-lyophilized lipase with cyclodextrins. The ability of cyclodextrins to form a host–guest complex to avoid product inhibition, which leads to the observed enhancement, has been proved by NOESY, COSY, ^{13}C and ^{1}H NMR.

Introduction

Hydrolases, especially lipases, represent the most frequently used enzymes in organic synthesis.[1] These natural catalysts have been shown to accept a broad range of substrates and are used as a useful tool for the synthesis of chiral molecules under non-aqueous conditions.[2] Their propensity for conducting transformations in organic solvents, safety, ease of handling and the mild conditions under which they operate (typically room temperature and around neutral pH) provide a further momentum in organic synthesis. Compared to aqueous solution, the use of an organic reaction medium can offer some interesting advantages. Among these advantages are: enhancement of the thermal stability of the enzyme, easy separation of the suspended enzyme from the reaction medium for recycling, increased solubility of the substrate, favorable equilibrium shift to synthesis over hydrolysis, elimination of undesired reactions caused by water and the generation of a *de novo* selectivity of the enzyme.[3] However, the use of enzymes in organic solvents has some drawbacks like their decreased catalytic activities, which are generally several orders of magnitude lower than in aqueous solution. In addition, many enzymatic reactions are prone to substrate or product inhibition, which deactivates the enzymes at higher substrate or product concentration, leading to a decrease in the reaction rate and enantioselectivity. In fact, keeping the substrate concentration at a low level through continuous addition can circumvent substrate inhibition, however, product inhibition is still difficult to control even by gradual removal of the product by physical means.[4] Substrate inhibition (*cf.* Fig. 1) is linked with the decrease of enzyme activity at higher concentrations of the substrate. This prevents the wasteful digestion of the abundant substrate and the accumulation of the product.

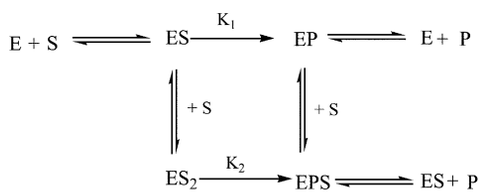

Fig. 1 Equilibrium associated with enzymes demonstrating substrate inhibition ($K_1 > K_2$).

At low concentration of the substrate, the enzyme–substrate complex (ES) regarded as a binary complex dominates, thus the rate of the reaction increases with increasing substrate concentration. As the substrate concentration increases further and further, the ternary complex ES_2 starts to appear and the rate of the reaction decreases. If enzymes which demonstrate substrate inhibition are used on an industrial scale, low substrate concentration and therefore a large-scale reaction is required.

Product inhibition (*cf.* Fig. 2) is linked with the decrease of enzyme activity at higher concentrations of the product. It can result from the binding of the product to the binary enzyme–substrate complex, reducing its catalytic activity, or through a decreased extent of product dissociation. The latter represents

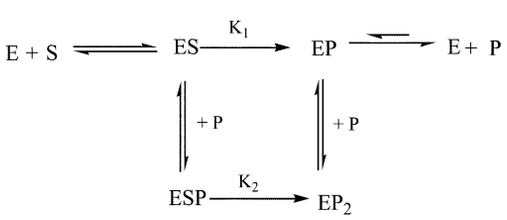

Fig. 2 Equilibrium associated with enzymes demonstrating product inhibition ($K_1 > K_2$).

† Electronic supplementary information (ESI) available: positive ion FAB mass spectrum of peracetylated cyclodextrin and details of gas-chromatographic separations of enantiomers. See http://www.rsc.org/suppdata/ob/b3/b301086d/

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product inhibition in its simplest form. The result of product inhibition is a decrease in the concentration of the reactive Michaelis complex, leading to a decrease in the reaction rate as more products are formed.

Several strategies have been explored to overcome the lower activity of enzymes in organic solvents to make them more appealing to organic chemists. These include the methods of enzyme preparation,[5] protein engineering,[6] chemical modification of the enzyme,[7] the control of the pH value,[8] co-lyophilization with lyoprotectants[9] and salts,[10] addition of water-mimicking compounds like formamide, glycol or DMF,[11] immobilization techniques,[12] imprinting with substrates and substrate analogues,[13] and cross-link crystallization.[14]

It has been reported that an additional improvement of the reaction conditions can be achieved by using certain additives, which might have a beneficial influence on the microenvironment of the enzymes and hence improve their catalytic activity and selectivity in organic solvents.[15]

Among these additives, crown ethers and especially thiacrown ethers have been successfully used to enhance the activity and the selectivity of *Pseudomonas cepacia* lipase (PSL) in the enantioselective formation and hydrolyses of carboxylic esters.[16] In fact, co-lyophilization of various enzymes with crown ether leads to enhancement of the activity of the enzyme and also increases the enantioselectivity in organic solvents.[17] It is conceivable to assume that the crown ether can bind to the enzyme in the co-lyophilization process leading to a possible solubilization of enzymes in organic solvents, and as a consequence, acceleration of the rate of transesterification reaction. Also, such binding can increase the flexibility of the enzymes in solvents, leading to enhancement of their activity and enantioselectivity *E*.[18] The enhancement of the activity of enzymes by a crown ether could also be attributed to its ability to facilitate the removal of water molecules from the active site upon substrate binding.[17]

Cyclodextrins (CDs), another class of macrocyclic structure, have been successfully used to improve enzyme activity and to increase the reaction rate and enantioselectivity in enzyme-catalyzed reactions in organic solvents.[17,18] The naturally occurring CDs are a family of cyclic α-(1,4)-linked glucose oligomers with six, seven, or eight glucose units, corresponding to α-, β-, and γ-cyclodextrin. They were discovered 100 years ago and produced from amylose by a highly selective enzymatic cyclization containing six, seven, or eight glucose monomers. They possess homogenous toroidal structures. The internal hydrophobic cavity and the external hydrophilic rim of the chemically modified CDs render them ideal for modeling enzyme–substrate binding,[19] drug delivery,[20] catalysis,[21] host–guest interaction,[22] chiral separation[23] and molecular recognition in self-assembled monolayers.[24] In the pharmaceutical industry, CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, and to increase their bioavailability and stability. In addition, they can be used to reduce or prevent gastro-intestinal (GI) or ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug–drug or drug–additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders. The ability of CDs to form complexes (Fig. 3) with a variety of organic compounds helps to alter the apparent solubility of the molecule, to increase stability in the presence of light, heat and oxidizing conditions and to decrease the volatility of compounds. CDs can also be used as processing aids to isolate compounds from natural sources and to remove undesired compounds such as cholesterol from food products.

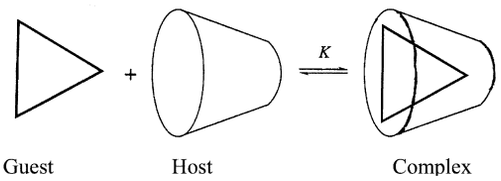


Fig. 3 Inclusion of a guest within a cyclodextrin host to form a complex.

These diverse applications of CDs prompted us to study their effects when they are used as regulators for the *Pseudomonas cepacia* lipase and additives to enhance the reaction rate and enantioselectivity in lipase-catalyzed transesterification of 1-(2-furyl)ethanol in organic solvent.

Results and discussion

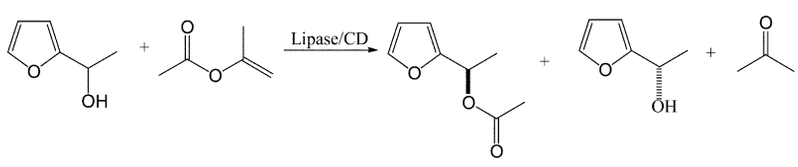
Co-lyophilized lipase with cyclodextrin-catalyzed transesterification of 1-(2-furyl)ethanol

Two methods of enzyme preparation have been investigated. The first consists of the lyophilization of *Pseudomonas cepacia* lipase (PSL), our model enzyme, from phosphate buffer (pH 6) (lyophilized enzyme only, without additives). The second enzyme preparation was a co-lyophilization of PSL with various CDs using the same phosphate buffer (co-lyophilized enzyme with CD). The effect of the addition of CDs on the activity and the enantioselectivity *E* of PSL-catalyzed transesterification of 1-(2-furyl)ethanol (**1**) in toluene was studied on a model example of lipase-catalyzed transesterification of 1-(2-furyl)ethanol using isopropenyl acetate as acyl donor (*cf.* Scheme 1).

Compared to the commercially available PSL (used as purchased), a slight decrease in activity has been observed for the PSL lyophilized from phosphate buffer alone (pH 6). This is probably due to partial denaturation of the enzyme during lyophilization. This slight decrease in activity was not observed when the enzyme was co-lyophilized with peracetylated cyclodextrin using the same phosphate buffer.

Compared to the lyophilized PSL-catalyzed transesterification of **1** in toluene, no enhancement effect on the enzyme enantioselectivity or the reaction rate was observed when the PSL was co-lyophilized with β-cyclodextrin. However, an increase in the enzyme enantioselectivity and catalytic rate was observed when the lipase was prepared in the presence of α- and γ-cyclodextrin at a 1 : 1 weight ratio of enzyme to cyclodextrin (*cf.* Figs. 4 and 5, Table 1).

It was concluded from this preliminary screening of native CD additives that there might be a relationship between the solubility of the CD in water and its ability to bind to the enzyme in the co-lyophilization process. In fact the solubility of native CDs in water at 25 °C varies from 14.5; 1.85; 23.2 [g/100 ml] for α-, β- and γ-cyclodextrin, respectively. The differences in their cavity diameter, which range from 4.7–5.3, 6.0–6.5, 7.5–8.3 Å,[25] respectively, suggest specific macrocycle–



Scheme 1 Co-lyophilized lipase with cyclodextrin-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol (**1**) using isopropenyl acetate (**2**) in toluene.

Table 1 Comparison between the lyophilized enzyme without additive (none) and the co-lyophilized PSL with native cyclodextrin (1 : 1) in catalyzing the enantioselective transesterification of **1** in toluene

Additives	Time/h	ee_S (%)	ee_P (%)	Conv. (%)	*E*
None (PSL lyophilized)	24	80	99	30	28
α-CD	24	65	97	45	>100
β-CD	24	12	94	12	33
γ-CD	24	98	44	68	>100

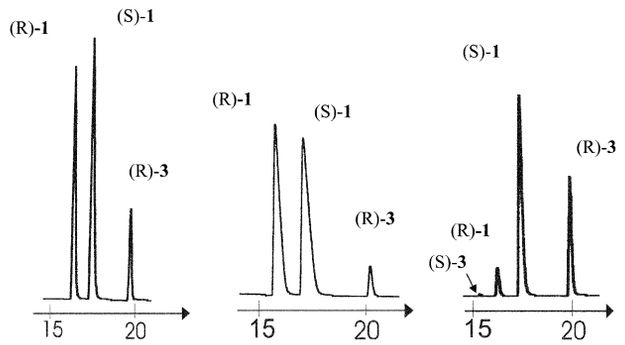


Fig. 4 Co-lyophilized PSL with native α-, β- and γ-cyclodextrin, respectively, at a 1 : 1 mol ratio of enzyme to cyclodextrin-catalyzed enantioselective transesterification of **1** in toluene.

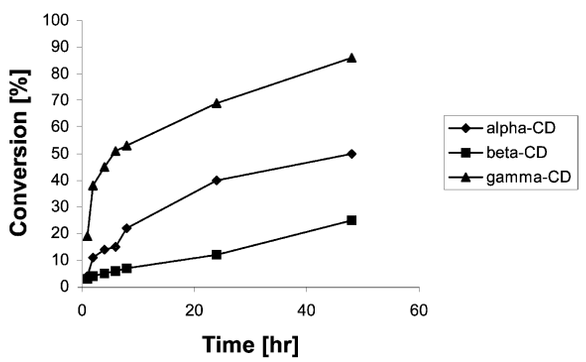


Fig. 5 Plot of the conversion against time of the co-lyophilized lipase with native cyclodextrins-catalyzed transesterification of **1** in toluene.

enzyme interactions. The poor solubility of the unmodified β-cyclodextrin in water results from the intramolecular hydrogen bonding of the secondary hydroxyl groups. Attempts to break this hydrogen-bond network by persubstitution using methyl, acetyl and hydroxypropyl groups improve their water solubility[26] and their physicochemical properties.[27] In fact hydroxyl groups in CDs could play an important role in the activation of lipase. To examine the effect of hydroxyl groups, several derivatized CDs have been used in the present study. Among the CD derivatives tested were permethylated α- and β-cyclodextrin, (3-hydroxypropyl)-γ-cyclodextrin, (2-hydroxypropyl)-α-, -β-, and -γ-cyclodextrin and peracetylated β-cyclodextrin.

In general, hydrophilic CD derivatives such as permethylated, hydroxyalkylated, and branched CDs have been used for the improvement of low solubility, dissolution rate, and bioavailability of poorly water soluble drugs.[28] Hydrophobic CDs like the ethylated and acylated derivatives have been used as sustained release carriers for water-soluble drugs.[29] Permethylated β-cyclodextrin has been used to enhance the enantioselectivity and reaction rate of the enzyme subtilisin Carlsberg suspended in organic solvent.[18] However, the introduction of an acetyl group into the free β-cyclodextrin is considered to be totally different from introduction of the methyl group from the point of view of bulkiness and hydrogen-bonding ability. Therefore peracetylated β-cyclodextrin is expected to exhibit different behavior, which can influence the enzyme's character if it is added prior to lyophilization of PSL in buffer. Also hydroxypropylated α-, β-, and γ-cyclodextrin have been used to increase the catalytic activity of α-chymotrypsin in organic solvent.[18]

In this investigation, it was found that permethylated α- and β-cyclodextrin as well as (2-hydroxypropyl)-α-cyclodextrin were efficient (reaction terminated after 24 h) in enhancing the catalytic activity of PSL-catalyzed transesterification of **1** when they were added prior to the lyophilization process in phosphate buffer. Peracetylated β-cyclodextrin (reaction terminated after 8 h) was the most effective among the CDs used as additives prior to lyophilization. The enhancement of the catalytic activity of PSL using these kinds of CD derivatives leads to an increase in the enantioselectivity and reaction rate of lipase-catalyzed transesterification of **1** in organic solvent. Water activity (aw) might also contribute to these phenomena; however, its effect was not investigated in this study.

Effect of concentration of additives on the PSL-catalyzed transesterification of 1

In comparison with other CDs used as additives in the PSL-catalyzed transesterification of **1**, the enhancement of the enantioselectivity and reaction rate was effective when using peracetylated β-cyclodextrin in co-lyophilized form with lipase.

Since the magnitude of the enzyme enhancement in organic solvents by macrocyclic compounds is dependent on the ratio of additive to enzyme,[16] three different concentrations have been used, 1 : 1; 1 : 2; 1 : 6 enzyme to cyclodextrin weight ratio.

When the peracetylated β-cyclodextrin was employed at a 1 : 6 weight ratio of enzyme to cyclodextrin, a significant activation of PSL was observed when compared to the data of the lyophilized PSL from buffer alone (*cf.* Fig. 6). The reaction was terminated in 8 h with 56% conv., > 99% ee_S, 80% ee_P, $E = 52$.

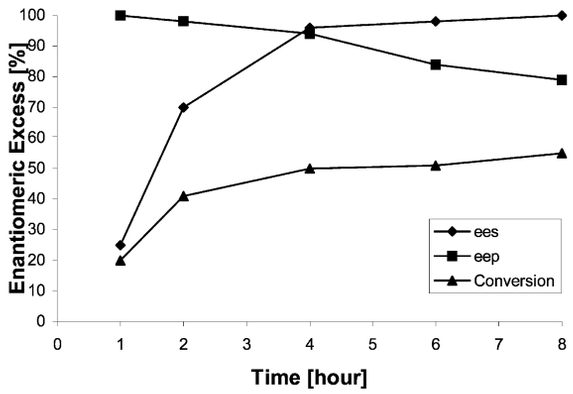


Fig. 6 Co-lyophilized lipase with peracetylated β-cyclodextrin (1 : 6 weight ratio)-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol in toluene.

When permethylated α- and β-cyclodextrin as well as (2-hydroxypropyl)-α-cyclodextrin were used prior to lyophilization (1 : 6 weight ratio enzyme to CD), the activity of PSL was increased and the enhancement of the reaction rate and enantioselectivity was significant, compared to the lyophilized enzyme without CD, but not as the peracetylated β-cyclodextrin. These results were a stimulus to study in detail the effect of peracetylated β-cyclodextrin additives upon the enzyme behavior. Results are summarized in Table 2.

Table 2 Co-lyophilized lipase with cyclodextrin-catalyzed enantioselective transesterification of **1** in toluene: Effect of the concentration ratio of enzyme to CD on the reaction improvement

Cyclodextrin (Weight ratio enzyme to CD)	ee_S (%) (*S*)-1	ee_P (%) (*R*)-3	Conv. (%)	*E*	Time/h
Mα-CD					
1 : 1	27.1	97	22	104	24
1 : 2	32	96	25	85	
1 : 6	>99	45	68	20	
Mβ-CD					
1 : 1	13	98	12	154	24
1 : 2	16	97	14	113	
1 : 6	86	95	48	130	
2HPα-CD					
1 : 1	13	99	11	>100	24
1 : 2	51	98	34	168	
1 : 6	94	92	50	81	
2HPβ-CD					
1 : 1	4	>99	3.5	>100	24
1 : 2	5	>99	4.5	>100	
1 : 6	15	99	13	>100	
2HPγ-CD					
1 : 1	4	99	3.5	>100	24
1 : 2	8	99	7	>100	
1 : 6	33	>99	25	>100	
3HPγ-CD					
1 : 1	2	99	2	>100	24
1 : 2	4	>99	3.7	>100	
1 : 6	31	>99	24	>100	

Table 3 Enantioselectivity enhancement of co-lyophilized lipase with peracetylated β-cyclodextrin-catalyzed transesterification of **1** in different organic solvents

Solvent	log P^a	Lyophilized[b] lipase	Peracetylated β-cyclodextrin co-lyophilisate[c]	Enhancement
Isooctane	4.5	$E^d = 46$	$E = 311$	6.7
THF	0.49	$E = 93$	$E = 580$	6.2
1,4-Dioxane	−1.1	$E = 28$	$E = 161$	5.8
n-Hexane	3.5	$E = 11$	$E = 40$	3.5
Acetonitrile	−0.33	$E = 99$	$E = 348$	3.5
Toluene	2.5	$E = 29$	$E = 51$	1.8

[a] P = partition coefficient; defined as the ratio of the concentration of a substance in two immiscible phases at equilibrium (octanol and water). [b] PSL lyophilized from phosphate buffer alone, pH 6. [c] Lyophilized from aqueous phosphate buffer containing peracetylated β-cyclodextrin at a 1 : 6 weight ratio of lipase to cyclodextrin. [d] E = enantioselectivity.

Influence of the organic solvent on the co-lyophilized enzyme behavior

The choice of the organic solvent for a lipase-catalyzed reaction is known to be very crucial in determining the enantioselectivity, which critically depends on the reaction medium employed. A trial to correlate the observed enantioselectivity with various solvents, depending on their log P (partition coefficient) has been performed. However, for this kind of enzyme preparation (co-lyophilized lipase with peracetylated β-cyclodextrin) and (lyophilized enzyme only), no correlation between the enzyme prepared, solvents and the enantioselectivity has been found (Table 3). For example (compared with the lyophilized lipase only), the enhancement of the enantioselectivity using co-lyophilized lipase with peracetylated β-cyclodextrin was 6.7 fold higher in magnitude in isooctane (log $P = 4.5$) while in THF (log $P = 0.49$) the enhancement was 6.2 fold higher in magnitude. Also in 1,4-dioxane (log $P = -1.1$) the enhancement in enantioselectivity was 5.8 fold higher in magnitude while in toluene (log $P = 2.5$) the enhancement was 1.8 fold higher in magnitude. In regard to the enantioselectivity (E), isooctane was the best solvent employed in the transesterification of **1** using the co-lyophilized lipase with peracetylated β-cyclodextrin. However, in regard to the reaction rate and enantioselectivity of both substrate and product, toluene was the best solvent employed in this kind of enzyme preparation.

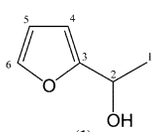
In this study, a reaction rate acceleration by CD in the absence of lipase was not observed. Only in the presence of lipase was an influence on the reaction noticeable. It is concluded from this finding that CDs do not catalyze the enantioselective transesterification of **1** by themselves, but they influence the enzyme activity, probably by changing the conformation of the enzymatic catalytic site. In an additional experiment, PSL was lyophilized from buffer and suspended in toluene, then CD was added next to the reaction mixture at the 1 : 6 weight ratio as before; a slight enhancement of the reaction rate and enantioselectivity was observed. This enhancement was much smaller than that observed when CD was used prior to lyophilization with PSL in phosphate buffer using the same weight ratio. The enhancement of the reaction rate of PSL-catalyzed transesterification of **1** was in the order 1 : 6 > 1 : 2 > 1 : 1.

Some conclusions can be derived from this experiment. First, CD must interact with the enzyme in a specific unknown way,

changing its conformation and hence influencing its behavior. This behavior is the result of the co-lyophilization of the enzyme with peracetylated β-cyclodextrin. Second, the excess of CD that did not bind to the enzyme remains in solution and acts as a host–guest complex. This might lead to understanding of the reasons for the enhancement of the reaction rate when using a 1 : 6 rather than using a 1 : 1 weight ratio of enzyme to CD. In fact, the ability of CDs to form a host–guest complex limits substrate as well as product inhibition.[17] The encapsulation of product within the cavity of a CD reduces the amount free in solution and, therefore, enhances the reaction rate. The ability of 1-(2-furyl)ethanol (**1**) as well as its ester (**3**) to be included in the peracetylated CD cavity was proved by complexation-induced shifts in ^{1}H as well as ^{13}C NMR. A preferential or enantioselective binding of substrate (**1**) with CD making one enantiomer more accessible by the enzyme, could be also contributing to the enhancement caused by CDs, allowing an enantioselective reaction with the acyl donor. When performing the esterification of **1** using pyridine as a catalyst (rather than lipase), acetyl chloride and using peracetylated β-cyclodextrin (600 mg) as additive, surprisingly, a poorly enantioselective reaction was observed and the ester formed did not show the expected 1 : 1 peak area ratio (the reaction has the following data: time = 7 h, ee_S = 0.26%, ee_P = 1.94%, Conv. = 12.0% and $E < 5$ and in 2 days, ee_S = 5.2%, ee_P = 31%, Conv. = 15% and $E < 5$). It is concluded that the inclusion of **1** in the peracetylated-β-cyclodextrin cavity has occurred in a preferential way. This preferential complexation of **1** by the peracetylated β-cyclodextrin cavity allows one enantiomer of **1** to be tightly attached by the CD while the other leaves the cavity faster to react with the acyl donor (acetyl chloride in this case), forming the corresponding ester. It was observed that (*R*)-**1** was the faster enantiomer in leaving the cavity while (*S*)-**1** was the slower one. The same effect was observed when using permethylated β-cyclodextrin (600 mg) as additive (the reaction has the following data: time = 7 hours, ee_S = 0.31%, ee_P = 16.7%, Conv. = 2.0% and $E < 5$ and in 2 days, ee_S = 0.6%, ee_P = 7.0%, Conv. = 8.0% and $E < 5$). However, the yield was very poor compared to the normal esterification reaction performed without CD additives. This supports the idea that the improvement of the reaction conditions of the co-lyophilized PSL with CD catalyzed transesterification of **1** in organic solvent was based on a combined effect of CD and enzyme, both of them contributing to the improvement of the reaction parameters. It is to be noted that other modified acylated CDs (*e.g.* perbutyrated CD) did not show any enhancement when used in a co-lyophilized form with lipase.

Trying to find proof of the binding of CD with PSL forming one block leading to a change in the enzyme behavior, SEM (scanning electron microscopy) and EDX (energy dispersive X-ray) of the co-lyophilized lipase with peracetylated β-cyclodextrin have been investigated.

Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) of co-lyophilized lipase with peracetylated β-cyclodextrin



(**1**)

In general, electron microscopy (EM) is one of the most useful tools in chemical science.[30] Scanning instruments now permit the imaging and the identification of nanoclusters consisting of only a few atoms. In order to shed some light on what has happened physically during the co-lyophilization of lipase with peracetylated β-cyclodextrin, scanning electron microscopy (SEM) has been used to compare the morphology of peracetylated β-cyclodextrin alone (Fig. 7a, b), lipase alone (Fig. 7c), co-lyophilized lipase with peracetylated β-cyclodextrin 1 : 1, 1 : 2 and 1 : 6 weight ratio (Fig. 7d,e,f, respectively). Comparing all the micrographs in Fig. 7, it was concluded that the morphology of lipase (Fig. 7c) has been totally changed upon mixing with peracetylated β-cyclodextrin in the co-lyophilized process. This change may lead to an improved dispersion of the lipase in the reaction medium, which will affect the reaction parameters.

Because of difficulties in classical elemental analysis, EDX-measurements (Fig. 8) were carried out. A typical EDX spectrum of enzyme alone is shown in Fig. 8a; also shown are spectra of peracetylated β-cyclodextrin alone (Fig. 8b) and co-lyophilized lipase with peracetylated β-cyclodextrin 1 : 6 weight ratio (Fig. 8c). Qualitative analysis confirmed the presence of carbon and oxygen in peracetylated β-cyclodextrin. Surprisingly, silicon (largest peak), oxygen, sodium, aluminium, calcium and iron were found in lipase only. After co-lyophilization of lipase with peracetylated β-cyclodextrin, EDX analysis confirmed the presence of carbon, oxygen, sodium, silicon and calcium, suggesting that lipase and peracetylated β-cyclodextrin are together in one particle and not separated. It was concluded from the SEM and EDX analysis that the addition of CD to the enzyme changes its morphology, leading to an improved dispersion of the lipase in the reaction medium which may contribute to the enhancement of the enantioselectivity and reaction rate in lipase-catalyzed transesterification reactions.

Evidence for the complexation of 1-(2-furyl)ethanol in peracetylated β-cyclodextrin

The ^{1}H NMR (400 MHz) signals of the hydroxyl group of **1**, recorded in the presence of peracetylated β-cyclodextrin, showed a downfield shift (0.17 ppm), which suggests the involvement of the OH group of **1** in complexation with CD (Table 4).

The neighboring proton at C_2 showed a downfield shift of 0.04 ppm, that of CH_3 was 0.02 ppm. The aromatic protons showed a different pattern in the presence of peracetylated β-cyclodextrin: the *ortho* and *para* hydrogens experienced a deshielding of 0.03 ppm while the *meta* hydrogen showed a deshielding of 0.02 ppm. Evidence of the inclusion of **1** in the cyclodextrin cavity is also provided by the observed ^{13}C NMR shift differences reported in Table 5 for a solution of the same concentration as used for recording the proton spectra.

The carbons of the aromatic ring were less sensitive to the presence of CD, only C_3 was more sensitive and showed a downfield shift of 0.08 ppm. Other carbons like C_4 showed an upfield shift of 0.01 ppm, C_5 showed a downfield shift of 0.01 ppm while C_6 showed no displacement. C_1 (methyl group) showed a downfield shift of 0.04 ppm while C_2 showed an upfield shift of 0.01 ppm.

In addition to the complexation-induced chemical shift of **1** in the presence of peracetylated β-CD, the NMR of peracetylated β-CD and its complexation-induced chemical shift difference can also provide information on the complexation geometry. The analysis of the shifts corresponding to the protons of peracetylated β-CD (host) can be related to the location of **1** (guest) in the CD cavity (*cf.* Table 6).

An upfield shift for the CD protons is expected when an aromatic guest molecule is located close to the protons in question since they are located in the shielding area of the aromatic ring of the guest. It is known that the H_3 and H_5 protons are located within the cavity of CDs, H_3 is situated near the larger rim, while H_5 is more deeply embedded in the cavity. H_6 protons are located at the narrower entrances and H_2, H_4 protons are

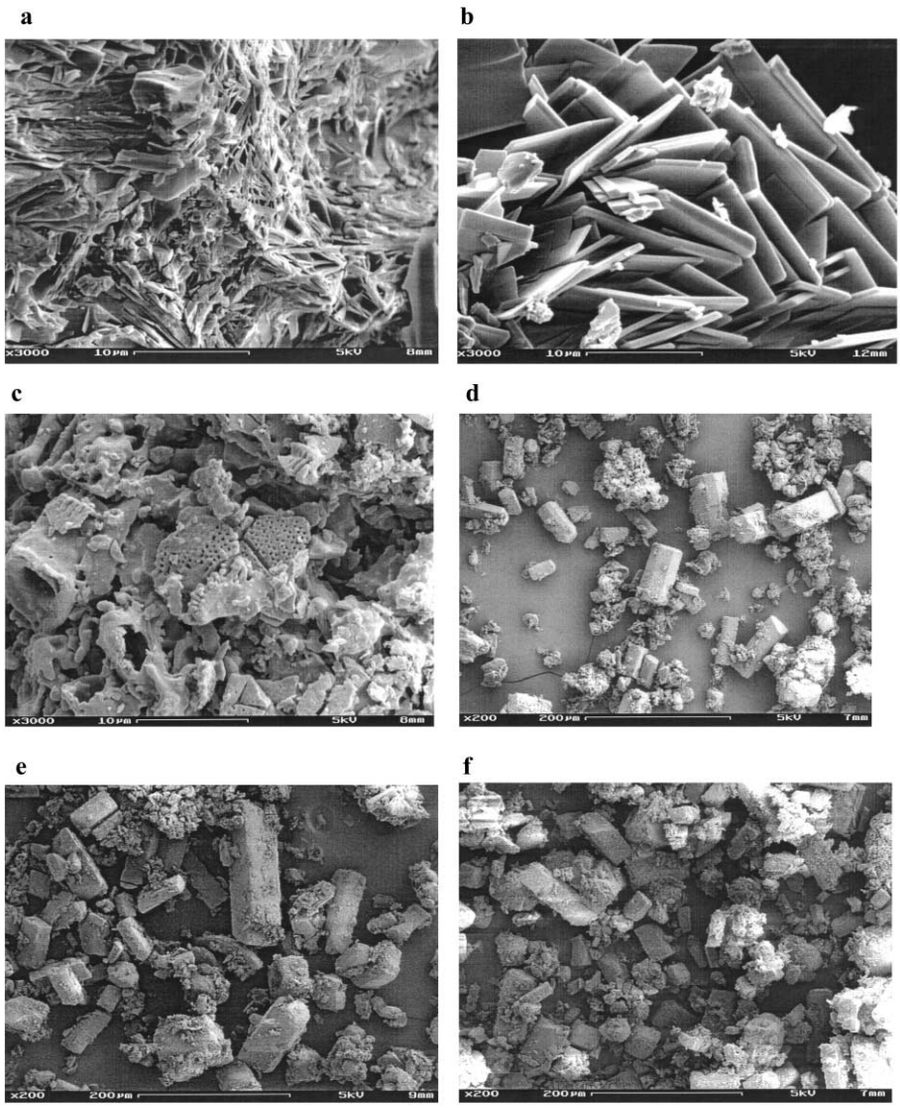


Fig. 7 An SEM study showing the morphology of the enzyme before and after co-lyophilization with peracetylated β-cyclodextrin. **a**: A typical SEM-micrograph of pure peracetylated β-cyclodextrin. **b**: An SEM-micrograph of a magnified part of Fig. 7a. **c**: A typical SEM-micrograph of the commercially available *Pseudomonas cepacia* lipase. **d**: An SEM-micrograph of co-lyophilized lipase with peracetylated β-cyclodextrin (1 : 1 weight ratio). **e**: A typical SEM-micrograph of the co-lyophilized lipase with peracetylated β-cyclodextrin (1 : 2 weight ratio). **f**: An SEM-micrograph of co-lyophilized lipase with peracetylated β-cyclodextrin (1 : 6 weight ratio).

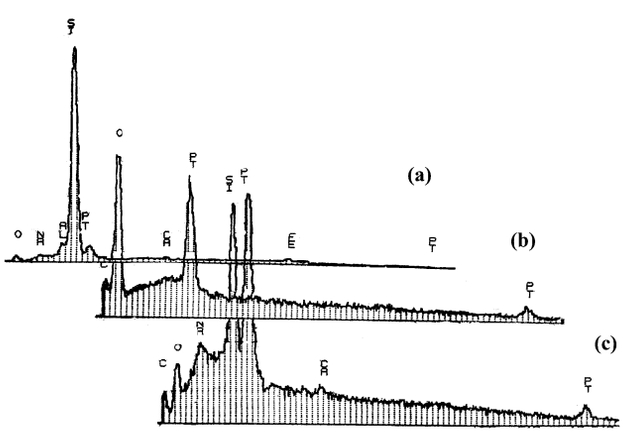


Fig. 8 An EDX study showing the difference between the compositions of (a) commercially available *Pseudomonas cepacia* lipase only, (b) peracetylated β-cyclodextrin only and (c) co-lyophilized lipase with peracetylated β-cyclodextrin.

located at the wider entrance. In the presence of **1**, the H_3, H_5 and H-1_6 protons were shifted upfield by 0.03 ppm, however, a much smaller downfield shift (0.02 ppm) was observed for H_4 while H_2 showed no displacement. H_1 showed a much higher downfield shift (0.04 ppm). These results suggest that the alcohol **1** is incorporated axially into the peracetylated β-CD cavity. Since the ^{1}H NMR spectroscopic data showed only small differences between the chemical shifts of the free and the complexed alcohol, it was decided to use more pronounced shifts to observe the interaction between furylethanol protons and those of the CD. This is based on ROESY spectra. The alcohol **1** in ROESY spectra showed intermolecular cross-peaks between the free hydroxyl group and both protons located at C_6 in the host as well as the methyl group of the acetyl moiety existing in the host. An interaction has been observed between the H-methyl of **1** and those of the acetyl group existing in the host. Only the aromatic protons located on C = 110 and C = 105 ppm (*ortho* and *meta*) in **1** showed a strong interaction with the H-3 and a weaker one with H-1 of the host, while the proton at C = 141 (*para*) did not show any interaction with the host. The proton of C–H in **1** showed an interaction with H-1, H-2, H-5 and H-6 of the host. No interaction has been observed between the aromatic protons of **1** and those of the 6th position in the host. This might give rise to a postulation about how **1** is included in the cavity of peracetylated β-CD (*cf.* Fig. 9)

Table 4 ^{1}H NMR of **1** and complexation-induced ^{1}H chemical shift difference (ppm)

	3H-CH_3	OH	H-C_2	H-C_4	H-C_5	H-C_6
1	1.32–1.33	2.47	4.59–4.64	6.00–6.01	6.03–6.05	7.05–7.15
1 + peracetylated β-CD	1.34–1.35	2.64	4.63–4.68	6.03–6.04	6.05–6.07	7.08–7.08
Chemical shift	0.02	0.17	0.04	0.03	0.02	0.03

Table 5 ^{13}C NMR of **1** and complexation-induced ^{13}C chemical shift difference (ppm)

	C_1	C_2	C_3	C_4	C_5	C_6
1	21.62	63.66	158.71	105.00	110.27	141.63
1 + peracetylated β-CD	21.66	63.64	158.79	104.99	110.28	141.63
Chemical shift	+0.04	−0.02[a]	+0.08	−0.01	+0.01	0.00

[a] Upfield displacements are negative.

Table 6 ^{1}H NMR of peracetylated β-CD and complexation-induced ^{1}H chemical shift difference (ppm)

	H-C_1	H-C_2	H-C_3	H-C_4	H-C_5	H1-C_6	H2-C_6	3H-CH_3
Peracetylated β-CD	5.17–5.24	4.99–5.03	5.59–5.63	3.72–3.76	4.30–4.33	4.79–4.82	4.40–4.44	1.87–1.95
Peracetylated β-CD + **1**	5.21–5.22	4.98–5.02	5.56–5.60	3.74–3.78	4.27–4.30	4.76–4.79	4.39–4.40	1.88–1.95
Chemical shift	+0.04	0.0	−0.03	+0.02	−0.03	−0.03	−0.01	+0.01

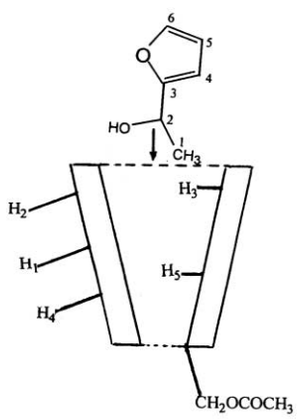
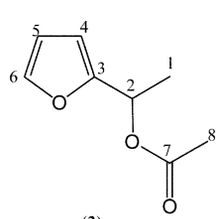


Fig. 9 A schematic representation of the inclusion of **1** in the cavity of peracetylated β-CD.

Evidence for the complexation of 3 in peracetylated β-cyclodextrin



(3)

The ester **3** can also be complexed in the CD to prevent product inhibition; the evidence for its complexation was quite similar to that for **1**.

The complexation-induced ^{1}H chemical shift differences of **3** in the presence of peracetylated β-cyclodextrin (not shown) were too small to assign for an inclusion. The interpretation of ^{13}C NMR shift changes was difficult due to the marked sensitivity of carbon shielding to even minor conformational distortions. The complexation-induced ^{1}H chemical shift difference recorded for peracetylated β-cyclodextrin in the presence of **3** (Table 7) showed a similar effect to that found in its spectra in the presence of **1**.

The ROESY spectra recorded for **3** in the presence of peracetylated β-cyclodextrin showed intermolecular cross-peaks between methyl protons of **3** and those of the acetyl moiety in the CD. The aromatic protons located on C = 110 and C = 105 ppm (*ortho* and *meta*) in **1** showed an interaction with the H-5 as well as H-3, H-6 and a weaker one with H-1 of the host, while the proton at C = 141 (*para*) did not show any interaction with the host. The proton of C–H in **3** did not show interaction with the host's protons.

It was concluded that the complexation of **3** with CD is nearly the same as it was with **1**.

Postulated mechanisms for the enhancement effects caused by the peracetylated β-cyclodextrin

As mentioned before, the improvement of the reaction conditions of the co-lyophilized PSL with cyclodextrin-catalyzed transesterification of **1** in organic solvent was based on a combined effect of the CD and the enzyme; both of them are contributing to the improvement of the reaction parameters. SEM and EDX have proved the effect of CDs on the enzyme morphology. This change in morphology of the enzyme might be responsible for the enhancement of the reaction rate observed in PSL-catalyzed transesterification of **1**. Other postulations are shown in the mechanism outlined below. It has been already proved that one aspect of the use of peracetylated β-cyclodextrin to alter the kinetics of an enzyme-catalyzed reaction involves the CD host selectively complexing a component of the reaction mixture (the alcohol **1** or the product **3**), hence reducing the amount free in solution. The complexation of **1** or **3** with peracetylated β-cyclodextrin has been indicated as described before by NMR techniques. Here a postulated mechanism based on the encapsulation of **1** or **3** inside the cavity of peracetylated β-cyclodextrin is reported. The first mechanism is based on the encapsulation of **1** inside the cavity of peracetylated β-cyclodextrin while the second mechanism is based on the encapsulation of **3** inside the cavity of peracetylated β-cyclodextrin.

When **1** is encapsulated in the cavity of a CD, an addition on the C=O occurs leading to the formation of the ester **3** and the free CD. The ester **3** itself acylates again the free CD, returning it again to the peracetylated β-cyclodextrin and regenerating the alcohol **1**; it is this cycle which removes the amounts of alcohol free in solution preventing substrate inhibition leading to the observed enhancement (*cf.* Fig. 10).

When **3** is the encapsulated component in the cavity of peracetylated β-cyclodextrin, the condensation occurs as outlined

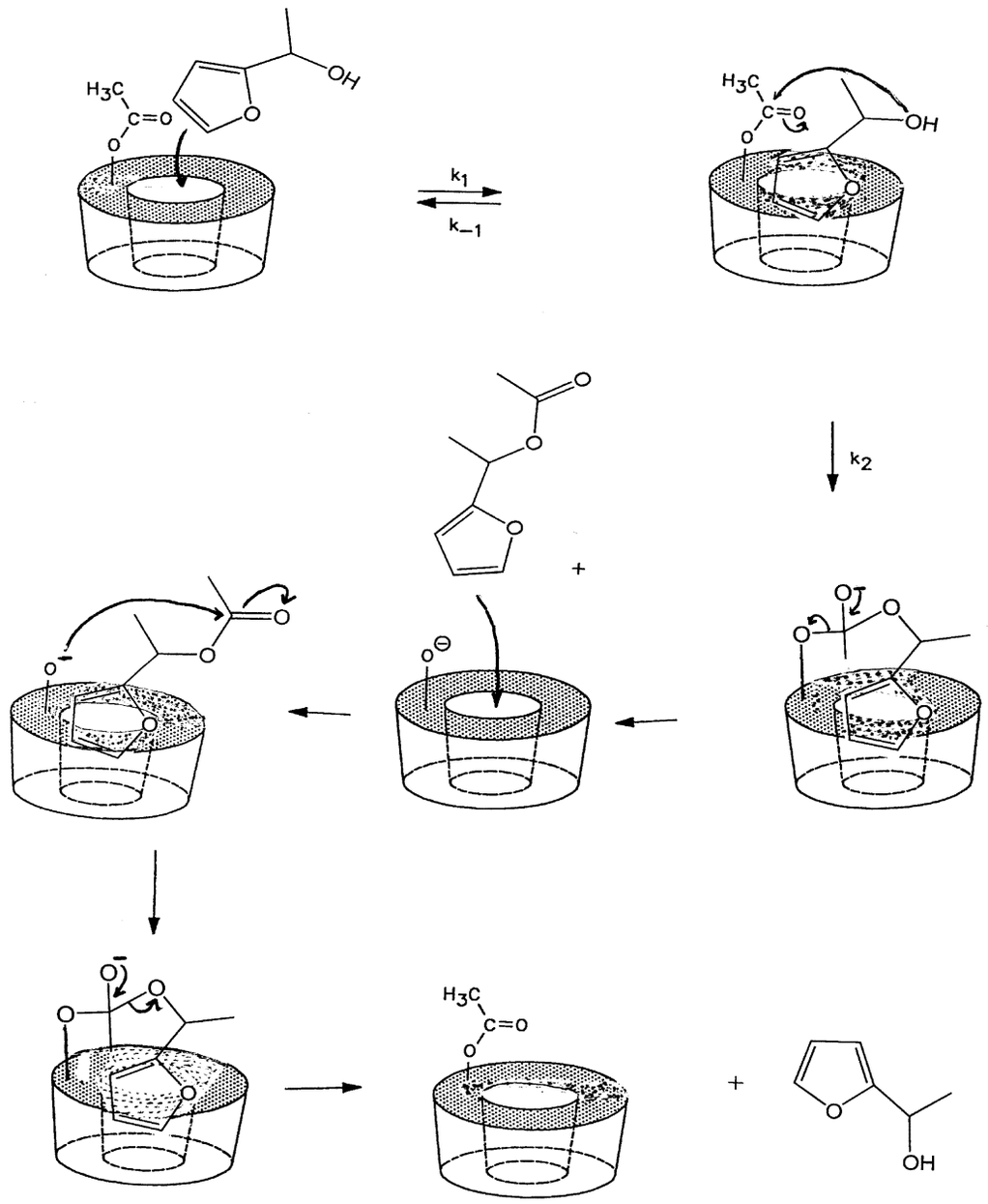


Fig. 10 The first postulated mechanism based on the encapsulation of **1** in peracetylated β-cyclodextrin.

Table 7 The complexation-induced ^{1}H chemical shift differences (ppm) resulting from inclusion of **3** in peracetylated β-cyclodextrin

	H-C_1	H-C_2	H-C_3	H-C_4	H-C_5	H1-C_6	H2-C_6	3H-CH_3
Chemical shift	+0.05	0.01	−0.02	+0.01	−0.02	−0.03	−0.01	+0.01

in Fig. 11. The acyl moiety of the ester **3** reacts with that of the peracetylated β-cyclodextrin affording the diketone and the free alcohol **1**. The free alcohol itself reacts again with the diketone by addition on C=O affording again the ester and the peracetylated β-cyclodextrin. This cycle prevents product inhibition and may contribute in the observed enhancement.

Trying to prove the second postulation, the lipase PSL was added to a mixture of 100 mg peracetylated β-cyclodextrin and 77 μl ester (*R*,*S*)-**3** dissolved in 3 ml toluene. The reaction was thermostated at 40 °C. Surprisingly, the alcohol (*R*)-**1** is formed after 4 days having the following data: ee_S [ester (*R*,*S*)-**3**] 41%; ee_P [alcohol (*R*)-**1**] 99.9%; Conv. = 29.1% and $E > 1000$. After 5 days, ee_S [ester (*R*,*S*)-**3**] 44%; ee_P [alcohol (*R*)-**1**] 99.9%; Conv. = 30.8% and $E > 1000$ (*cf.* Fig. 12). The formation of the alcohol (*R*)-**1** can be due to the condensation shown in the second postulation (*cf.* Fig. 11) or the lipase PSL contains some water which can itself hydrolyze the ester (*R*,*S*)-**3**; however, the second assumption is not favored.

Materials and methods

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer AMX600 FT NMR (Karlsruhe, Germany) operating at 600.130 MHz and a sample temperature of 300 K. Chemical shifts (δ) are given in ppm relative to TMS as internal standard. The rotating Frame Overhauser Experiments (ROESY) were performed using the pulse program roesyprtp with an applied mixing time of 225 ms. Scanning electron micrographs (SEM) and energy dispersive X-ray analysis (EDX) were performed on a DSM 962 scanning electron microscope (Zeiss, LEO)

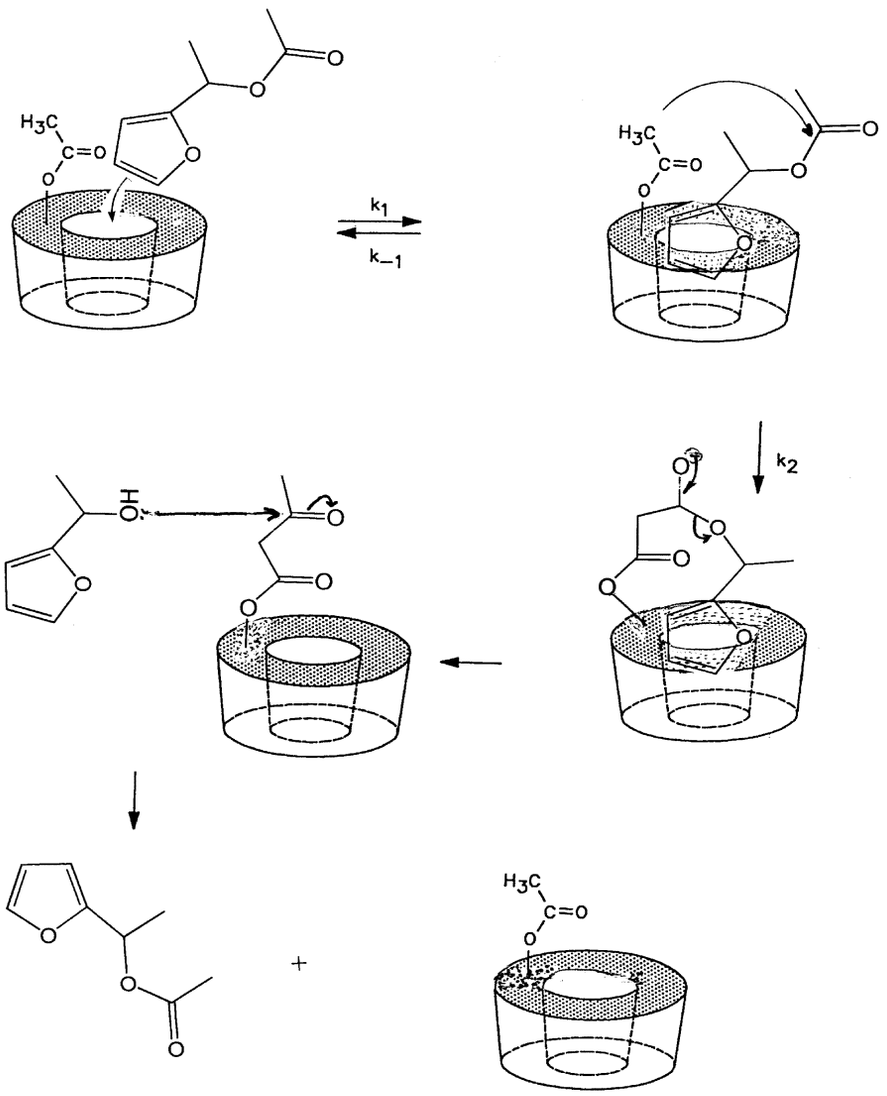


Fig. 11 The second postulated mechanism based on the encapsulation of **3** in peracetylated β-cyclodextrin.

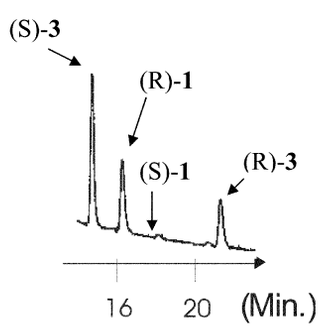


Fig. 12 Gas-chromatographic separation of the enantiomers of both substrate (*R*,*S*)-**3** and product (*R*)-**1** resulting from the reaction of peracetylated CD and ester (*R*,*S*)-**3** in the presence of PSL in toluene, t = 5 days, ee_S [ester (*R*,*S*)-**3**] 44%; ee_P [alcohol (*R*)-**1**] 99.9%; Conv. = 30.8% and $E > 1000$.

equipped with a DX-4 X-ray detection system by EDAX. This consists of an energy dispersive Si(Li)-detector with an active area of 10 mm² and the edx software package. Micrographs were recorded detecting secondary electrons generated by a probe current of 168 pA, whereas a 623 pA probe current was applied for carrying out elemental analysis by EDX.

Chemicals and enzymes

All chemicals were purchased from Fluka (Switzerland). Lipase from *Pseudomonas cepacia* (PSL) was a gift from Amano (Nagoya, Japan). α-, β-, and γ-cyclodextrin, methylated β-cyclodextrin (average molar substitution = 1.8), methylated α-cyclodextrin (average molar substitution = 1.8) and (3-hydroxypropyl)-γ-cyclodextrin (average molar substitution = 0.6) were obtained from Wacker-Chemie GmbH (Burghausen, Germany). (2-Hydroxypropyl)-α-, -β-, and -γ-cyclodextrin (average molar substitution = 0.6) were purchased from Fluka. The peracetylated β-cyclodextrin was synthesized as described before.[31]

Co-lyophilization of PSL with cyclodextrin derivatives

100 mg *Pseudomonas cepacia* lipase (PSL) was dissolved (2 mg/1 µl) in 20 mM phosphate buffer (pH 6.0) and lyophilized for 48 h (control). The co-lyophilization of lipase with cyclodextrin was performed by the same method, except that the cyclodextrin was added prior to lyophilization with different ratios of lipase to cyclodextrin (1 : 1; 1 : 2; 1 : 6, w/w). After lyophilization, the enzyme preparation was dried under vacuum and stored at −18 °C for further use.

Lipase-catalyzed transesterification of 1-(2-furyl)ethanol

All reactants (alcohol, ester) were stored over activated molecular sieves 4 Å.

56 mg (0.5 mmol) racemic 1-(2-furyl)ethanol and 108.8 mg (1 mmol) isopropenyl acetate were dissolved in 3 ml organic solvent in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath to 40 °C. Then, a 100 µl sample of

the reaction mixture was withdrawn for GC analysis (t = 0 of sample). Afterward, 100 mg of lyophilized lipase or lipase co-lyophilized with 100, 200 or 600 mg cyclodextrin was added. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was diluted by 100 µl toluene. The reaction progress was monitored qualitatively by thin layer chromatography. An aliquot of the supernatant was used for the enantioselective GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. Substrate (*S*)-**1** and product (*R*)-**3** were distilled and the recovered cyclodextrin was purified and analyzed.

Enantioselective gas-chromatographic analysis

Enantioselective analysis was performed on a gas chromatograph (Hewlett Packard 580, Waldbronn, Germany) equipped with a flame ionization detector (FID). The chiral stationary phase heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin, 20% (w/w) was dissolved in PS 86 (Gelest, ABCR GmbH & Co., Karlsruhe, Germany) and coated on a 25 m × 0.25 mm (id) fused silica capillary column (0.25 µm film thickness) according to the literature procedure.[32] The analytical conditions were: injector temperature, 200 °C; FID temperature, 250 °C; oven temperature 70 °C for the simultaneous separation of enantiomers of both substrate and product. Hydrogen was used as the carrier gas (40 KPa column head pressure). The retention times of (*S*)-**3**, (*R*)-**1**, (*S*)-**1**, (*R*)-**3** were 14.7, 15.5, 16.8, 20.2 min, respectively. The substrate and product were identified by using a GC/MSD-system HP 6890/5973 (Hewlett Packard, Waldbronn, Germany) equipped with an HP 7683 autosampler. The enantiomeric excess of both substrate and product as well as conversion and enantioselectivity E were determined by the computer program available on the internet http://www.orgc.TUGraz.at/orgc/programs/selectiv/selectiv.htm, developed by Faber *et al.*[33]

Conclusion

The utility of cyclodextrins as additives in enzymatic reactions has been demonstrated on a model example of *Pseudomonas cepacia* lipase (PSL)-catalyzed transesterification of 1-(2-furyl)-ethanol in organic solvent. We tried to shed some light on the factors contributing to the enhancement of the reaction rate and enantioselectivity. The results suggest that co-lyophilization with cyclodextrin can be a useful method for the activation of enzymes in organic solvents.

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References

1 U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis*, Wiley-VCH: Weinheim, Germany, 1999.
2 (*a*) A. Ghanem and V. Schurig, *Chirality*, 2001, **13**, 118–123; (*b*) A. Ghanem and V. Schurig, *Tetrahedron: Asymmetry*, 2003, **14**, 57–62; (*c*) A. Ghanem, C. Ginatta, Z. Jiang and V. Schurig, *Chromatographia*, 2003, in press; (*d*) A. Ghanem and V. Schurig, *Monatsh. Chem.*, 2003, in press; (*e*) A. Ghanem, PhD thesis, University of Tübingen, Germany, 2002.
3 A. M. Klibanov, *Trends Biochem. Sci.*, 1989, **14**, 141.
4 K. Faber, *Biotransformations in Organic Chemistry*, Springer Verlag: Heidelberg, Germany, 1996.
5 H. Noritomi, Ö. Almarsson, G. L. Barletta and A. M. Klibanov, *Biotechnol. Bioeng.*, 1996, **49**, 87–92.
6 M. T. Reetz and K. E. Jaeger, *Biocatalysis*, 1999, **200**, 31–57.
7 M. T. Babonneau, R. Jacquier, R. Lazaro and P. Viallefont, *Tetrahedron Lett.*, 1989, **30**, 2787.
8 Z. Yang, D. Zacherl and A. J. Russell, *J. Am. Chem. Soc.*, 1993, **115**, 12251–12257.
9 K. Dabulis and A. M. Klibanov, *Biotechnol. Bioeng.*, 1993, **41**, 566–571.
10 Y. L. Khmelnitsky, S. H. Welch, D. E. Clark and J. S. Dordick, *J. Am. Chem. Soc.*, 1994, **116**, 2647–2648.
11 H. Kitaguchi and A. M. Klibanov, *J. Am. Chem. Soc.*, 1989, **111**, 9272.
12 M. Reslow, P. Adlercreutz and B. Mattiasson, *Eur. J. Biochem.*, 1988, **172**, 573.
13 J. O. Rich and J. S. Dordick, *J. Am. Chem. Soc.*, 1997, **119**, 3245–3252.
14 T. Theil, *Tetrahedron*, 2000, **56**, 2905–2919.
15 Y. Takagi, J. Teramoto, H. Kihara, T. Itoh and H. Tsukube, *Tetrahedron Lett.*, 1997, **37**, 4991–4992.
16 J. Bross, A. J. Visser, J. F. Engbersen, W. Verboom, A. V. Hoek and D. N. Reinhoudt, *J. Am. Chem. Soc.*, 1995, **117**, 12657–12663.
17 J. B. Harper, C. J. Easton and S. F. Lincoln, *Curr. Org. Chem.*, 2000, **4**, 429–454.
18 K. Griebenow, Y. D. Laureano, A. M. Santos, I. M. Clemente, L. Rodriguez, M. W. Vidal and G. Barletta, *J. Am. Chem. Soc.*, 1999, **121**, 8157–8163.
19 (*a*) M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer: Berlin, 1978; V. T. D'Souza and M. L. Bender, *Acc. Chem. Res.*, 1987, **20**, 146–152; (*b*) Y. Murakami, J. Kikuchi, Y. Hisaedda and O. Hayashida, *Chem. Rev.*, 1996, **96**, 721–758.
20 B. Pfannemuller and W. Burchard, *Macromol. Chem.*, 1969, **121**, 1.
21 H. Ye, W. Tong and V. T. D'Souza, *J. Am. Chem. Soc.*, 1992, **144**, 5470.
22 (*a*) H. Ohvo and J. P. Slotte, *Biochemistry*, 1996, **35**, 8108; (*b*) T. Anderson, M. Sundahl, G. Westman and O. Wennerstorm, *Tetrahedron Lett.*, 1994, **35**, 7103.
23 (*a*) V. Schurig, *J. Chromatogr. A*, 1994, **666**, 111–129; (*b*) V. Schurig and H. P. Nowotny, *J. Chromatogr.*, 1988, **441**, 155–163.
24 M. T. Rojas, R. Koniger, J. F. Stoddart and A. E. Kaifer, *J. Am. Chem. Soc.*, 1995, **117**, 336–343.
25 Wacker-Chemie GmbH, Burghausen, Germany.
26 C. R. Wescot and A. M. Klibanov, *J. Am. Chem. Soc.*, 1993, **115**, 1629–1631.
27 K. Nakamura, M. Kinoshita and A. Ohno, *Tetrahedron*, 1994, **50**, 4681–4690.
28 L. C. Hansch and D. Elkins, *Chem. Rev.*, 1971, **71**, 525–616.
29 F. Terradas, M. Tenston-Henry and P. A. Fitzpatrick, *J. Am. Chem. Soc.*, 1993, **115**, 390–396.
30 D. M. Veiga, J. Diaz and F. Ahsan, *J. Pharm. Sci.*, 1998, **87**, 891.
31 A. Ghanem and V. Schurig, *Tetrahedron: Asymmetry*, 2001, **12**, 2761–2766.
32 A. Dietrich, B. Mass, W. Messer, G. Bruche, V. Karl, A. Kaunzinger and A. Mosandl, *High Resolut. Chromatogr.*, 1992, **15**, 590–593.
33 W. Kroutil, A. Kleewein and K. Faber, *Tetrahedron: Asymmetry*, 1997, **8**, 3251–3261.