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Activity enhancement of pig liver esterase in organic solvents by colyophilization with methoxypolyethylene glycol: kinetic resolution of alcohols

Stephan Ruppert and Hans-Joachim Gais *

Institut für Organische Chemie, Rheinisch-Westfälische Technische Hochschule Aachen, Professor-Pirlet Straße 1, 52056 Aachen, Germany

Abstract: Colyophilization of pig liver esterase with methoxypolyethylene glycol gave a catalyst, PLE/MPEG, which showed an enhanced activity in organic solvents. The PLE/MPEG catalyzed transesterification of the alcohols *rac*-**1a–d** with vinyl propionate in toluene proceeded with good to high selectivities. The addition of up to 1% of water to the reaction mixture resulted in a significant increase in enantioselectivity. Immobilization of PLE/MPEG for the batch-wise resolution was accomplished by its spontaneous adsorption on an ultrafiltration membrane. © 1997 Elsevier Science Ltd

Introduction

Pig liver esterase (PLE) is one of the most versatile hydrolases for asymmetric synthesis.[1–3] The enzyme has been widely used for the enantioselective hydrolysis of dicarboxylic acid diesters and acylated diols as well as racemic esters in water.[4] However, despite their obvious synthetic potential, transesterifications catalyzed by PLE have never gained the importance of those catalyzed by lipases.[3,5,6] This is due to the low activity and stability of PLE in organic solvents. Attempts to solve this problem were met with various degrees of success. Transesterification with an aqueous solution of PLE confined to the pores of a porous support in organic solvents[7] suffers from a poor reproducibility and a low operational stability of the enzyme.[8] The covalent attachment of methoxypolyethylene glycol (MPEG) to PLE gave MPEG–PLE which showed an enhanced activity in transesterifications in organic solvents.[9] The necessity of a covalent modification of PLE, however, detracts somewhat from this approach. Furthermore, the activity and stability of MPEG–PLE in organic solvents is not satisfactory. We now report that by a simple colyophilization of PLE and MPEG, a catalyst, PLE/MPEG, is obtained whose activity and stability in organic solvents is such as to allow for the kinetic resolution of alcohols via transesterification.

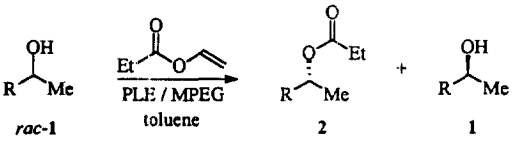
Results and discussion

MPEG had been covalently attached to PLE in aqueous solution by hoping that the polymer chains would retain their hydration in water immiscible organic solvents and thus provide the enzyme with exactly the amount of water necessary for its activity.[10] During our investigations of MPEG–PLE[9] the question arose, however, whether it would not suffice to simply blend PLE and MPEG together. Previous studies of other hydrolases had revealed a positive effect of added MPEG or polyethylene glycol upon the enzyme activity.[11–14] Thus, PLE (30 mg, 200 U/mg) was colyophilized with $MPEG_{5000}$ (1 g) from an aqueous solution for 48 h at 0.09 mbar to give PLE/MPEG as a white powder, having a protein content of 3% and an estimated water content of 1–2%. PLE lost only 10–15% of its activity upon colyophilization with MPEG as determined by the hydrolysis of ethyl butyrate in water. This loss is perhaps due to a partial denaturation of the enzyme during the lyophilization. In organic solvents PLE/MPEG forms a fine suspension which most likely contains aggregates of PLE complexed by

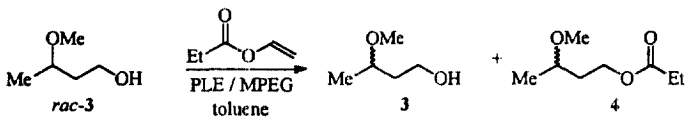
* Corresponding author. Email: Gais@RWTH-Aachen.de

MPEG. Gratifyingly, in organic solvents PLE/MPEG showed an activity which was at least as high as that of MPEG–PLE.[8] It is of importance for the activity of PLE/MPEG in organic solvents that the amount of MPEG in the reaction mixture exceeds the solubility of the polymer in the solvent used. Otherwise the aggregates between PLE and MPEG are destroyed and the activity of PLE decreases to that of the free enzyme.

The secondary and primary alcohols *rac*-**1a–d** and *rac*-**3**, respectively, were submitted to a PLE/MPEG catalyzed transesterification with vinyl esters by using water-saturated toluene, containing *ca* 0.03% water (v/v), as the solvent (Scheme 1). The selectivities, which are expressed in apparent E-values,[15,16] varied from poor for the primary alcohol *rac*-**3**[7] to moderate for the secondary alcohols *rac*-**1a–d** under these conditions (Table 1).



a : R = $CH_2CH_2CH{=}C(CH_3)_2$, **b** : R = $(CH_2)_3CH(CH_3)_2$, **c** : R = $(CH_2)_5CH_3$, **d** : R = CH_2Ph



Scheme 1

PLE exhibited a preference for the (*R*) configurated secondary alcohol and, thus, gave preferentially the alcohols **1a**,[17,18] **1b**,[19–21] **1c**,[18,22] **1d**[23,24] and the esters **2a**, **2b**, **2c**[25] and **2d**.[25] Table 1 reveals that the activity of PLE but not the selectivity of the transesterification is altered by the admixture of MPEG. PLE/MPEG was easier to handle than PLE and MPEG–PLE, and the reproducibility of the transesterification was considerably better in the case of PLE/MPEG. We studied the influence of several parameters on the activity of PLE/MPEG and the selectivity of the transesterification in case of the alcohol *rac*-**1d**. An increase of the temperature from 25 to 40°C led to an increase in enzyme

Table 1. PLE/MPEG catalyzed acylation of the alcohols *rac*-**1a–d** and *rac*-**3** in toluene (0.03% added water, v/v) with vinyl propionate

substrate	enzyme (U/mL)[a]	time (d)	conversion (%)	alcohol ee (%)	ester ee (%)	E
rac-**1a**	PLE (66)	10	11	10	80	10
rac-**1a**	PLE/MPEG (80)	10	54	78	67	11
rac-**1b**	PLE (80)	10	5	6	73	7
rac-**1b**	PLE/MPEG (80)	10	47	54	62	7
rac-**1c**	PLE (80)	10	5	3	82	10
rac-**1c**	PLE/MPEG (80)	10	50	61	62	8
rac-**1d**	PLE (80)	4	4	5	91	22
rac-**1d**	PLE/MPEG (80)	4	53	85	76	19
rac-**3**	PLE (20)	3	17	n.d.	40	2.5
rac-**3**	PLE/MPEG (20)	2[b]	49	36	38	3

[a] Units per mL of the reaction mixture. [b] In h.

Table 2. PLE/MPEG (50 U/mL) catalyzed acylation of *rac*-**1d** in toluene (0.03% added water, v/v) with various vinyl esters

acyl donor	time (d)	conversion (%)	alcohol ee (%)	ester ee (%)	E
vinyl acetate	10	36	44	79	13
vinyl propionate	10	48	70	80	18
vinyl butyrate	10	50	83	83	27

[a] Units per mL of the reaction mixture.

activity and to a faster inactivation. The selectivity remained nearly unchanged. The results obtained in isooctane and 1,1,1-trichloroethane as solvents did not differ significantly from the results with toluene as solvent. A change of the acyl donor from vinyl acetate to vinyl propionate and vinyl butyrate resulted in an increase in the activity of the enzyme as well as of the selectivity of the acylation (Table 2). Similar results were obtained with *rac*-**1a–c** as substrates.

It turned out, however, that the water content of the system is the most important factor, influencing strongly the enantioselectivity and the activity of the enzyme in the transesterifications. In a series of transesterifications with PLE/MPEG in toluene of an initial water content of 0.03% (v/v), various amounts of water were added to the reaction mixture (Figure 1). At a water content of the system higher than *ca* 0.5% a second liquid phase was formed on the surface of the flask, containing besides water an unspecified amount of the enzyme and the polymer. An increase of the water content from 0.03% to 1.06% (v/v) led to a sharp increase of the selectivity of the transesterification of *rac*-**1c**.[26] In addition, during the reaction the selectivity varied, depending on the amount of water present.

At initial water contents of 0.03% and 1.06% the selectivity either remained unchanged or varied little whereas at a water content of 0.46% it dropped sharply. The activity of the enzyme on the other hand decreased with an increasing water content and, in addition, increased during the course of the reaction. Similar observations were made in the case of the acylation of *rac*-**1a**, *rac*-**1b**, *rac*-**1d** and *rac*-**3**. Thus, at a water content of 1.06% the apparent E-values for the acylation of *rac*-**1a–d** and *rac*-**3** increased to 100 and 10, respectively, and these values remained nearly unchanged as the reaction progressed. The phenomena of the time dependency of the selectivity and activity can at least in part be attributed to a decrease of the water content during the course of the transesterification because of a water consuming enzymatic hydrolysis of the acyl donor. The formation of propionic acid was easily demonstrated by GC analysis. A reversibility of the transesterification cannot account for the decrease in selectivity. The ee-value of **1c** increased at conversions higher than 50% and reached values of 98% or higher, depending on the amount of enzyme used. According to a recent study of the molecular flexibility of lyophilized subtilisin in organic solvents, the increase in the enantioselectivity of PLE/MPEG with an increasing water content could be ascribed to a higher enzyme flexibility because of an increasing enzyme hydration.[27] However, the results of previous studies of hydrolases in organic solvents suggest that other factors may be important too.[6,28–33] Furthermore, the influence of the formation of a second liquid phase at higher water concentrations upon the selectivity and the activity of PLE/MPEG is not yet fully clear.

Despite the problem of a decreasing water content and, thus, of a decreasing selectivity during the transesterification, the PLE/MPEG catalyzed transesterification could be applied to the kinetic resolution of the alcohols *rac*-**1a–d** on a 5–10 mmol scale with satisfactory results (Table 3).

In the batch-wise resolution the catalyst PLE/MPEG could conveniently be immobilized by adsorption on a polyaramide ultrafiltration membrane. The membrane, whose pores were filled with water, was placed in the flask, containing PLE/MPEG as a suspension in toluene. Within minutes a clear solution was formed. Small droplets, which consisted of water, MPEG and PLE, appeared on the surface of the membrane. No PLE remained in the organic phase. Subsequently, a toluene solution of the substrate and the acyl donor was added. After a reaction time of 24 h, the organic phase was replaced by another solution of the substrate and the acyl donor in toluene. This procedure

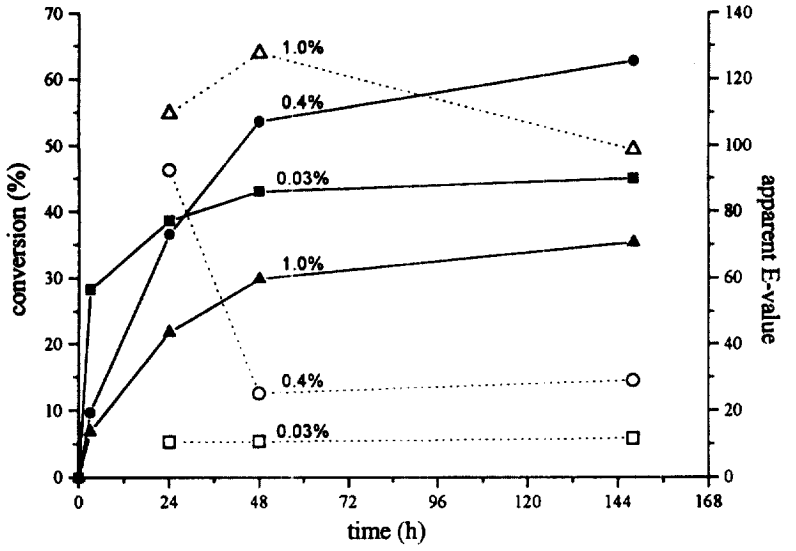


Figure 1. PLE/MPEG catalyzed acylation of *rac*-**1c** with vinyl propionate in toluene under variation of the amount of water added to the reaction mixture; squares: 0.03%; circles: 0.4%; triangles: 1% (v/v); solid symbols (•, ■ ,▲): conversion, open symbols (○ ,□ ,△): apparent E-value.

Table 3. PLE/MPEG (83 U/mL) catalyzed acylation of *rac*-**1a–d** on a 5–10 mol scale in toluene (0.5% added water, v/v) with vinyl propionate

substrate	time	conversion	alcohol		ester	
	(d)	(%)	yield (%)	ee (%)	yield (%)	ee (%)
rac-**1a**	5	57	94	96	97	72
rac-**1b**	10	39	89	58	91	89
rac-**1c**	10	46	88	68	90	80
rac-**1d**	3	53	96	97	93	85

was repeated several times. In this manner, starting with 2.56 g (20 mmol) of *rac*-**1a**, 1.39 g of **2a** (88% ee) and 1.35 g of **1a** (53% ee) were obtained by using 3 mg of PLE (600 U) (*cf.* Figure 2). Here too we observed an increase in selectivity in the presence of a small amount of water in the reaction medium. During the first two batches the apparent E-value was *ca* 100. Subsequently the E-value dropped to 20–25 and remained nearly constant over several batches. We think that also in this case the decrease in selectivity is due to the consumption of water by hydrolysis of the acyl donor, resulting in a reduction of the total water content of the reaction mixture. During the first two batches the formation of large amounts of propionic acid could be detected by GC analysis. Within the next three batches the formation of the acid stopped almost completely. The activity of the enzyme increased within the first two batches as the water content decreased. These results match those obtained in absence of the membrane. The operational stability of PLE/MPEG under these conditions was quite high. Even after a storage of PLE/MPEG confined to the surface of the membrane for 1 week at 4°C in toluene the loss in enzyme activity was less than 5%.

Conclusion

Colyophilization of PLE with MPEG gave PLE/MPEG which showed a significantly enhanced activity and stability in transesterifications in organic solvents as compared to PLE. Depending on the water content, high apparent E-values were recorded in the acylation of the secondary alcohols

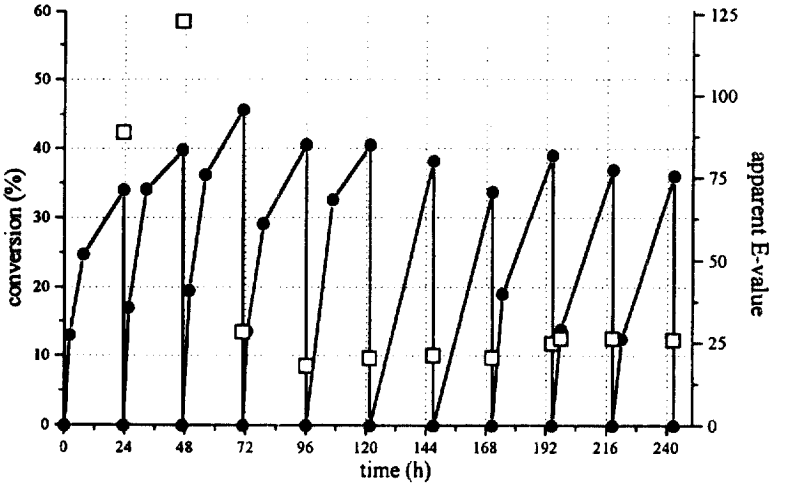


Figure 2. Acylation of *rac*-**1a** with vinyl propionate in toluene catalyzed by PLE/MPEG adsorbed on an ultrafiltration membrane; circles: conversion in %; squares: apparent E-value.

rac-**1a–d** catalyzed by PLE/MPEG in toluene. The selectivities are comparable to those obtained by using lipases, which show, however, a higher activity in the case of secondary alcohols. Because of the strong dependence of the selectivity of the enantiomer differentiation and the activity of PLE/MPEG on the water content, further investigations are necessary to find an effective means to adjust this experimental parameter to an optimal value.

Experimental

General remarks

Chemical shifts are given in ppm relative to Me_4Si: δ=0.00 as the internal standard. Peaks in the ^{13}C NMR spectra are denoted as "u" for carbons with zero or two attached protons or as "d" for carbons with one or three attached protons, as determined from the ATP pulse sequence. Enzymatic reactions were monitored by GC using a CP-Sil-8 column. PLE (EC 3.1.1.1, 200 U/mg) was purchased from Boehringer Mannheim as a suspension in 3.2 M NH_4SO_4. The enzyme was desalted by ultrafiltration (cut-off 30 kDa) under ice cooling and lyophilized prior to use. The activity of PLE was determined by hydrolysis of ethyl butyrate in aqueous buffer solution (pH 8.0, 25°C). For the batch-wise transesterification a UF-PA-20H membrane (Hoechst AG) was used. The membrane was kept in water and washed with toluene before use. Enantiomer compositions were determined by GC analysis with an octakis-(2,3-*O*-dipentyl-6-*O*-methyl)-γ-cyclodextrin column (25 m×0.25 mm) (Lipodex γ-6-Me) (Macherey–Nagel), a permethylated β-cyclodextrin column (25 m×0.25 mm) (CP-Chirasil-Dex-CB) (Chrompack) and an octakis-(2,6-di-*O*-pentyl-3-*O*-butyl)-γ-cyclodextrin column (25 m×0.25 mm) (Lipodex E) (Macherey–Nagel). The carrier gas was hydrogen at 100 kPa except otherwise noted. Retention times are given in min. Column chromatography was performed with E. Merck silica gel 60 (230–400 mesh). Lyophilizations were performed with a Christ alpha 2–4.

Determination of enantiomer composition

rac-**1a** and *rac*-**2a**: Lipodex γ-6-Me, split 1:40, 60°C (20 min) → 100°C (10°C/min) (5 min): t_R (**1a**)=10.2, t_R (*ent*-**1a**)=12.1, t_R (*ent*-**2a**)=15.7, t_R (**2a**)=18.6 min. *rac*-**1b** and *rac*-**2b**: Lipodex γ-6-Me, 50 kPa, split 1:40, 60°C (20 min) → 100°C (10°C/min) (5 min): t_R (**1b**)=15.5, t_R (*ent*-**1b**)=16.5, t_R (*ent*-**2b**)=22.7, t_R (**2b**)=23.6. *rac*-**1c** and *rac*-**2c**: Lipodex γ-6-Me, split 1:40, 70°C (5 min) → 110°C (10°C/min) (5 min) → 140°C (10°C/min) (5 min): t_R (**1c**)=5.5, t_R (*ent*-**1c**)=5.7, t_R (*ent*-**2c**)=7.6, t_R (**2c**)=8.1. *rac*-**1d** and *rac*-**2d**: CP-Chirasil-Dex-CB, split 1:40, 100°C (5 min) → 120°C (10°C/min)

(5 min) → 140°C (10°C/min) (5 min): t_R (**1d**)=7.6, t_R (*ent*-**1d**)=7.8, t_R (*ent*-**2d**)=9.9, t_R (**2d**)=10.3. *rac*-**3** and *rac*-**4**: Lipodex E, split 1:40, 50°C (5 min) → 100°C (10°C/min) (5 min), t_R (**3**)=7.0, t_R (*ent*-**3**)=7.3, t_R (**4**)=7.8, t_R (*ent*-**4**)=7.9.

Preparation of PLE/MPEG

PLE (30 mg) was desalted by ultrafiltration (cut-off 30 kDa) under ice cooling. The remaining aqueous solution of PLE (100 mL) was mixed with $MPEG_{5000}$ (1.0 g) and the mixture stirred magnetically for 2 h. The resultant mixture was frozen in liquid nitrogen and lyophilized (0.009 mbar, 48 h) to give PLE/MPEG as a white powder. PLE/MPEG had a specific activity of 170–190 U as determined by the hydrolysis of ethyl butyrate in water under standard conditions. PLE/MPEG lost none of its activity upon storage at 4°C for several weeks. The water content of PLE/MPEG was estimated to be 1–2% by drying the same amount of MPEG at 120°C and 2.5×10^{-4} mbar after lyophilization and differential weighting.

Preparation of rac-***2a–d***

The alcohol (23 mmol) was mixed with a solution of the freshly distilled anhydride (47 mmol) and pyridine (0.16 g, 2 mmol). After stirring the mixture at room temperature for 24 h, it was poured into ice-water. The organic layer was separated and the aqueous layer extracted with ether. The combined organic phases were stirred with saturated aqueous $NaHCO_3$ for 12 h. The organic layer was separated, dried (Na_2SO_4) and concentrated under vacuum. The ester was purified by chromatography (*n*-hexane:EtOAc, 3:1).

(±)-6-Methyl-5-heptene-2-yl-propionate rac-***2a***

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.09 (tm, J=7.0 Hz, 1H), 4.90 (m, 1H), 2.31 (q, J=7.5 Hz, 2H), 2.02 (m, 2H), 1.68 (d, J=1 Hz, 3H), 1.58 (s, 3H), 1.51 (m, 2H), 1.21 (d, J=6.5 Hz, 3H), 1.13 (t, J=7.5 Hz, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 174.1 (u), 132.0 (u), 123.6 (d), 70.4 (d), 36.1 (u), 28.0 (u), 25.7 (d), 24.1 (u), 20.0 (d), 17.6 (d), 9.2 (d).

(±)-6-Methylheptan-2-yl-propionate rac-***2b***

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.91 (m, J=6.5 Hz, 1H), 2.29 (q, J=7.5 Hz, 2H), 1.56 (m, 3H), 1.30 (m, 2H), 1.17 (m, 8H), 0.90 (d, J=7.0 Hz, 6H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 174.2 (u), 70.8 (d), 38.8 (u), 36.2 (u), 28.0 (u), 27.9 (d), 23.2 (u), 22.6 (d), 20.0 (d), 9.3 (d).

Transesterification of rac-***1a–d*** *and* rac-***3*** *with PLE and PLE/MPEG*

The alcohol (1 mmol) and the vinyl ester (4 mmol) were dissolved in toluene (10 mL) previously saturated with water. Subsequently PLE or PLE/MPEG (200–800 U) was added. The reaction mixture was stirred magnetically at room temperature.

Transesterification of rac-***1a–d*** *and* rac-***3*** *with PLE/MPEG under variation of the water content*

The alcohol (1 mmol) and the vinyl ester (4 mmol) were dissolved in toluene (10 mL) containing 0.03% (v/v) of water. Subsequently PLE/MPEG (700 U) and the calculated amount of water were added. The reaction mixture was stirred magnetically at room temperature.

Enzymatic transesterification of rac-***1a–d*** *on a preparative scale*

rac-**1a**: The alcohol *rac*-**1a** (1.0 g, 7.8 mmol), vinyl propionate (1.95 g, 19.5 mmol), toluene (38.5 mL, 0.03% water, v/v) and water (193 µl, 0.5%, v/v) were combined. Subsequently PLE/MPEG (610 mg, 3200 U) was added to the mixture, which was stirred magnetically at room temperature. The reaction was stopped at 57% conversion (5 d) by silica gel filtration of the mixture. Chromatography (*n*-hexane:EtOAc, 3:1) of the residue gave **1a** (0.40 g, 94%) of 96% ee, $[\alpha]_D^{22}$ +14.9 (*c* 2, EtOH) and **2a** (0.81 g, 98%) of 71% ee, $[\alpha]_D^{22}$ −6.0 (*c* 2.7, EtOH).

rac-**1b**: Following the above procedure, *rac*-**1b** (1.0 g, 7.7 mmol), vinyl propionate (1.93 g, 19.3 mmol) and PLE/MPEG (610 mg, 3200 U) in toluene (38.5 mL), containing water (193 µl, 0.5%, v/v),

gave at 39% conversion (10 d) **1b** (0.54 g, 89%): 58% ee, $[\alpha]_D^{22}$ +4.8 (*c* 3, $CHCl_3$) and **2b** (0.51 g, 91%): 89% ee, $[\alpha]_D^{22}$ −3.3 (*c* 3.3, $CHCl_3$).

rac-**1c**: Following the above procedure, *rac*-**1c** (1.0 g, 7.7 mmol), vinyl propionate (1.93 g, 19.3 mmol) and PLE/MPEG (610 mg, 3200 U) in toluene (38.5 mL), containing water (193 µl, 0.5%, v/v), gave at 46% conversion (10 d) **1c** (0.45 g, 83%): 68% ee, $[\alpha]_D^{25}$ +8.7 (*c* 2.7, ether) and **2c** (0.56 g, 85%): 80% ee, $[\alpha]_D^{25}$ −4.3 (*c* 2.5, ether).

rac-**1d**: Following the above procedure, *rac*-**1d** (1.0 g, 7.3 mmol), vinyl propionate (1.75 g, 18 mmol) and PLE/MPEG (610 mg, 3200 U) in toluene (37 mL), containing water (185 µl, 0.5%, v/v), gave at 53% conversion (3 d) **1d** (0.45 g, 96%): 97% ee, $[\alpha]_D^{22}$ +40.2 (*c* 1.15, benzene) and **2d** (0.69 g, 93%): 85% ee, $[\alpha]_D^{22}$ −9.7 (*c* 1.08, ether).

Batch-wise transesterification of rac-***1a*** *with PLE/MPEG*

rac-**1a** (0.256 g, 2 mmol) and vinyl propionate (0.50 g, 5 mmol) were dissolved in toluene (10 mL), containing 0.03% (v/v) of water. Subsequently PLE/MPEG and the water-saturated membrane (*ca* 4 cm^2) were added. Within 15 min, PLE and most of the MPEG were adsorbed on the membrane. The mixture was stirred magnetically at room temperature for 24 h. The organic phase was removed by a syringe, and a solution of *rac*-**1a** (0.256 g, 2 mmol) and vinyl propionate (0.50 g, 5 mmol) in toluene (10 mL) were added. This procedure was repeated 10 times, and the organic phases obtained were combined. Work-up and chromatography (*n*-hexane:EtOAc, 3:1) of the residue gave **2a** (1.39 g, 98%) of 88% ee and **1a** (1.35 g, 89%) of 53% ee.

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