Second Generation Sol-Gel Encapsulated Lipases: Robust Heterogeneous Biocatalysts

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Abstract: The original procedure for the encapsulation of lipases in sol-gel materials produced by the fluoride-catalyzed hydrolysis of mixtures of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$ has been improved considerably. This involves higher enzyme loading, variation of the alkylsilane precursor, and the use of additives such as isopropyl alcohol, 18-crown-6, Tween 80®, methyl-β-cyclodextrin and/or KCl. A dramatic increase in enzyme activity is observed. The sol-gel lipase immobilizates are also excellent catalysts in the kinetic resolution of chiral alcohols and amines, recycling without any substantial loss in enantioselectivity and a residual activity of 70% being possible even after 20 reaction cycles.

Keywords: asymmetric catalysis; enzyme catalysis; immobilization; kinetic resolution; sol-gel processes

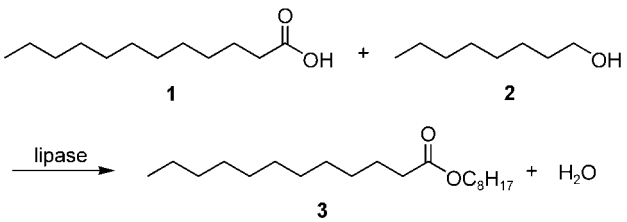
Introduction

Lipases (EC 3.1.1.3) are the most used enzymes in synthetic organic chemistry, catalyzing the hydrolysis of carboxylic acid esters in aqueous medium or the reverse reaction in organic solvents.[1,2] They are structurally characterized by a so-called lid. When hydrophobic substrates interact with the lipase, the lid opens and thus exposes the active site (serine) in a process called interfacial activation.[2] The catalytic triad, composed of aspartate, histidine and serine, then induces the formation of a short-lived acyl-enzyme intermediate which reacts with water or an alcohol liberating the acid or the ester, respectively. Other nucleophiles such as hydrogen peroxide or amines can also be used, which results in the formation of acid peroxides or amides, respectively. Numerous examples involving enantioselectivity have been reported, either in the desymmetrization of prochiral (*meso*-type) substrates or in the kinetic resolution of chiral compounds.[1,2]

In order for lipases or other enzymes to be industrially viable, they need to be recycled and reused.[3] This can be accomplished by employing membrane reactors, special solvent systems or immobilization techniques.[4] Moreover, cross-linking of lipases (CLECS)[5] and cross-linking of lipase aggregates[6] have also been reported. So far most efforts have focused on immobilization, of which many approaches are known including adsorption on appropriate supports or covalent attachment to such materials as well as encapsulation in polymers or in sol-gel materials.

Sol-gel encapsulation has proven to be a particularly easy and effective way to immobilize enzymes.[7] Following isolated reports describing specific examples, it was the seminal work of Avnir and co-workers which led to the generalization of this technique.[7,8] It involves the acid- or base-catalyzed hydrolysis of tetraalkoxysilanes $Si(OR)_4$[9] in the presence of an enzyme. Mechanistically, the silane-precursor undergoes hydrolysis and cross-linking condensation with formation of an SiO_2 matrix in which the enzyme is encapsulated. This type of encapsulation works well for a number of enzymes.[7,8] However, when we attempted to apply the method to lipases, materials were obtained which showed disappointingly low enzyme activities, as measured by the rate of the model reaction involving the esterification of lauric acid (**1**) by *n*-octanol (**2**) in non-dried isooctane as solvent.[10] Only 5–10% activity relative to the traditional use of the respective lipase powder was observed, equivalent to relative rates of 0.05 to 0.1.

Reasoning that the micro-environment in SiO_2 may be too polar, we tested the use of mixtures of $Si(OCH_3)_4$



Scheme 1.

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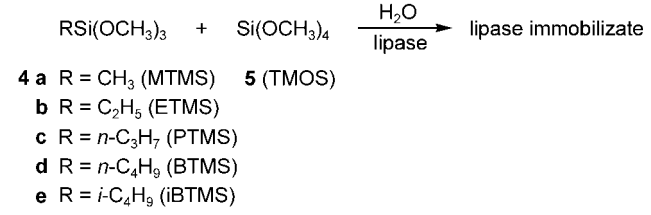
and alkylsilanes of the type $RSi(OCH_3)_3$ having non-hydrolyzable alkyl groups (R).[10] We speculated that the silicon oxide matrix, which is now hydrophobic, could facilitate or simulate interfacial activation of the entrapped lipase. Indeed, dramatically improved relative lipase activities typically amounting to 200–800% were observed in the model reaction, which corresponds to an enhancement of relative enzyme activity by a factor ranging from 2 to 8 with respect to the traditional use of the corresponding lipase powder (lyophilizate).[10,11] Relative activity is defined as [v(immobilized lipase)/v(commercial lipase)], where v is the initial rate of the reaction in each case. A pronounced increase in thermal stability was also observed. In most cases the optimal ratio of $RSi(OCH_3)_3$ to $Si(OCH_3)_4$ turned out to be about 5:1, although it was not possible to present an experimental protocol which is completely general. Usually $CH_3Si(OCH_3)_3$ was used as the precursor and polyvinyl alcohol (PVA) as an additive, the latter possibly acting as a stabilizer of the lipase.[10,11] Other research groups have employed these lipase immobilizates and/or developed similar materials which are also based on hydrophobic alkylsilanes.[12,13] An interesting extension of our method pertains to the use of additional porous solid supports during the sol-gel process.[14] This type of "double immobilization" involves binding of the lipase-containing gels in the pores of the solid support (e.g., silicates of the type SIRAN® or Celite®) as gelation occurs and results in higher thermal stability and activity (up to a factor of 88). It should be noted that sol-gel encapsulation is crucial in both variants since conventional adsorption on hydrophobic silicates or on SIRAN® alone affords poor catalysts.[10]

Successful recycling of first generation sol-gel lipase immobilizates was demonstrated in a series of experiments using the model reaction, 30 consecutive runs resulting in only 12–15% decrease in activity.[11,15] Although these early immobilizates are commercially available,[16] they have not been utilized often in enantioselective processes.[10a,13] Immobilization was found to increase enantioselectivity in a few examples of kinetic resolution, but general conclusions were not possible. In the present study we describe a highly improved version of sol-gel encapsulation of lipases, as well as the use of these new immobilizates as heterogeneous biocatalysts in a select number of enantioselective reactions. Optimization was achieved by higher enzyme loading, variation of the alkyl group in the silane precursor and the use of appropriate additives.



Scheme 2.

Results and Discussion

Optimization of the Sol-Gel Lipase-Immobilizates

In our original work we had observed an "alkyl effect", i.e., enhancement of lipase activity upon using $RSi(OCH_3)_3$ in the series methyl < ethyl < *n*-propyl < *n*-butyl.[11] It appeared that enhanced hydrophobicity in the silicon oxide matrix correlates with increased enzyme activity. Higher thermal stability and activity were postulated to result from multipoint interaction through hydrogen bonding as well as ionic and hydrophobic interactions (van der Waals), which can be schematized as shown in Figure 1. Hydrophobic interactions could result in a type of interfacial activation. It may be that the lipase is conformationally arrested in the matrix in a "lid-opened" and therefore active form.

Considerably larger alkyl groups at silicon cause problems because this slows down the rate of gelation to a point where the process becomes unpractical. However, small branched alkyl groups at silicon had been neglected. In the present study we therefore concentrated on a comparison of the *n*-butyl- and isobutylsilanes (**4d** and **4e**, respectively) in the sol-gel process, hoping to learn whether branching in the alkyl group of the silane has any effect on the activity of the lipase. Moreover, the potential effects of additional additives such as crown ethers (e.g., 18-crown-6), β-cyclodextrin derivatives (e.g., methyl-β-cyclodextrin), salts (e.g., KCl) and the surfactant Tween 80® were

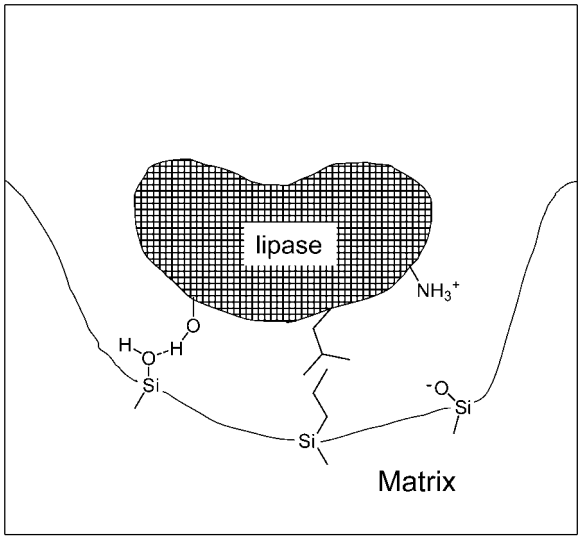


Figure 1. Schematic view of non-covalent interactions between the gel matrix and the lipase.

studied. Previous studies by Reinhoudt,[17] Griebenow,[18] Dordick[19] and Xu,[20] respectively, had shown that lipases show higher activities and occasionally enhanced stereoselectivities when used in the presence of such additives. Moreover, in yet another study Kazlauskas demonstrated that the presence of a small amount of isopropyl alcohol is beneficial.[21] Usually interactions of the additive and the lipase on the basis of hydrogen bonds were postulated, although in some cases ambiguities as to the origin of the activating effect remain.

We have performed extensive optimization,[22] but only the most important results are presented here. In preliminary experiments it was shown that the presence of isopropyl alcohol during the sol-gel process does indeed exert a beneficial effect. Therefore, isopropyl alcohol and PVA were included in all experiments. Moreover, considerably higher loadings were strived for.

The degree of enzyme loading can in principle be controlled by adjusting the amount of enzyme per unit of silane used in the sol-gel process, maximum loading being the goal because this results in the least amount of the heterogeneous catalyst weight-wise subsequently needed in catalysis. However, this feature had not been optimized in the first generation sol-gel lipase immobilizates. Indeed, rather low loading was used (1 – 5 mg of protein per gram of silicate matrix), and early attempts to double the loading led to lower enzyme activities.[10b] The disadvantage of having to use large amounts of heterogeneous catalysts becomes accentuated when performing the sol-gel encapsulation in the presence of a solid support such as glass beads of the type SIRAN® or other porous silicates such as Celite®.

In the present study initial experiments were performed with the lipase B from *Candida antarctica* (CaLB; Chirazyme L-2®/Roche). Using either BTMS (**4d**) or iBTMS (**4e**) as the silane precursors in the presence of isopropyl alcohol and PVA, it was possible to increase loading considerably without any loss in activity or specific activity. These are defined as μmol product per minute per gram of gel [μmol/min · g gel] and μmol product per minute per gram of protein [μmol/min · g protein], respectively. In fact, intrinsic lipase activity as measured by the specific activity turned out to be *higher* at such high loadings, the latter typically ranging between 50 and 600 mg of protein per gram of silicate matrix. The reason for this positive effect is currently unclear. Each additional additive was then studied separately at high loading and in the presence of isopropyl alcohol and PVA. Optimization was restricted to the variation of the amount of additional additive. In typical experiments the possible influence of 18-crown-6 was first investigated. Accordingly, in a standard sol-gel reaction 3 mmol of silane (**4** + **5** in a 5 : 1 ratio), isopropyl alcohol, PVA, buffer (0.1 M TRIS/HCl; pH 7.5) and different amounts of 18-crown-6 were shaken in the presence of CaLB and a basic catalyst (NaF). Figure 2 shows the results of a series of experiments using iBTMS (**4e**) which demonstrate that the amount of 18-crown-6 is crucial to success. Enzyme activity in the model reaction is more than doubled when employing 0.5 mmol of this additive. When calculating the specific activity, which considers the degree of loading, an even greater value results, amounting to a factor of 3.4. Interestingly, when using greater amounts of the additive, activity decreases. Similar results were obtained when using BTMS (**4d**)/TMOS (**5**) as the silane precursors (Table 1). Thus, in this particular case it makes little difference whether the isobutyl- or *n*-butyl-derived silicate is employed, although the former seems to lead to slightly better results.

In further experiments the standard protocols were extended to include the presence of 50 mg of Celite® as a porous solid support, which led to two interesting observations (Table 1). In the case of the iBTMS-derived sol-gel, the "double immobilization" results in increased enzyme activity, both in the absence or presence of 18-crown-6. Thus, the best CaLB-immobilizate makes use of 18-crown-6 *and* Celite®, this combination increasing the relative activity to a factor of 30.5. Table 1 also reveals that KCl (optimally 0.75 mmol) as an additive likewise increases enzyme activity, although the effect is not as pronounced. In contrast, methyl-β-cyclodextrin or Tween 80® showed no activating effect. Most of the experiments were carried out several times, and reproducibility turned out to be remarkably good (± 20% of the values for activity).

The same types of experiments were then performed with the lipases from *Pseudomonas fluorescens* (PfL, Amano AK), *Burkholderia cepacia* (BcL, Amano PS), *Mucor miehei* (MmL, Chirazyme L-9®/Roche), *Aspergillus niger* (AnL, Amano AS), *Candida rugosa* (CrL, Chirazyme L-3®), *Candida rugosa* type VII (CrL type

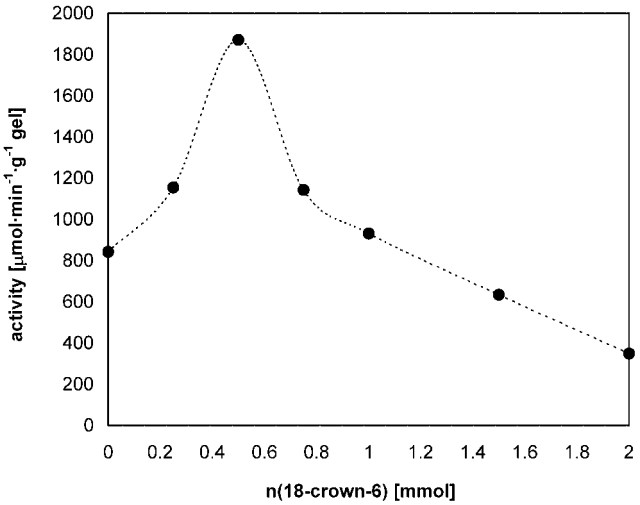


Figure 2. Influence of different amounts of 18-crown-6 as an additive (see text).

Table 1. Activity of sol-gel encapsulated CaLB in the model reaction involving **1/2** compared to the use of non-immobilized lipase powder (activity = 244 μmol/min · g; specific activity = 458 μmol/min · g protein). In all cases a **4/5** ratio of 5:1 (total 3 mmol) was used in the presence of PVA, isopropyl alcohol and CaLB (125 mg of lyophilizate).

Entry	Silane	Additives	Activity [μmol/min · g gel]	Specific Activity [μmol/min · g protein]	Relative Activity
1	**4d**	none	767	2584	5.6
2	**4d**	Celite®[a]	711	1684	3.7
3	**4d**	18-crown-6[b]	1088	3912	8.5
4	**4d**	Celite®[a]/18-crown-6[b]	1080	3313	7.2
5	**4d**	KCl[c]	751	1975	4.3
6	**4d**	Celite®[a]/KCl[c]	490	2403	5.2
7	**4e**	none	843	1259	2.7
8	**4e**	Celite®[a]	1076	2631	5.7
9	**4e**	18-crown-6[b]	1871	4252	9.3
10	**4e**	Celite®[a]/18-crown-6[b]	2729	13997	30.5
11	**4e**	KCl[c]	1639	2927	6.4
12	**4e**	Celite®[a]/KCl[c]	1288	2807	6.1

[a] 50 mg. [b] 0.5 mmol. [c] 0.75 mmol.

VII, Sigma), *Thermomyces lanuginosa* (TIL, Novo Nordisk), lipase from pig pancreas (PpL, Chirazyme L-7®) and *Penicillium roqueforti* (PrL, Fluka). The results are summarized in Table 2. Although all of the above additives were tested in every case, positive (activating) effects were not always found (which are not listed). In two extreme cases none of the additives resulted in increased lipase activity (namely MmL and CrL type VII), although the advantage of high loading was in fact achieved here (Table 2, entries 33–36 and 45–48) as in all other cases.

Some of the results deserve additional comment, especially in view of the fact that a general protocol for all lipases cannot be expected. The difference between BTMS (**4d**) and iBTMS (**4e**) is often small (e.g., entries 33/35), but in some cases substantial (e.g., entries 17/25). With respect to additives, the use of 18-crown-6 or Tween 80® leads most often to positive effects, whereas KCl or methyl-β-cyclodextrin are only useful in isolated cases. At this stage we prefer not to attempt to explain the various trends on a molecular basis. Empirically, in going from the first generation sol-gel lipase immobilizates to the second generation variants, the specific and relative activities increase by an *additional* factor ranging between 2 and 8. The numbers are even more revealing when comparing the new gels with the traditional use of non-immobilized lyophilizates (Table 2), the most dramatic case pertaining to Tween 80®-activated TIL in a BTMS-derived gel on Celite® which results in a relative activity factor of 1391 (entry 64). These observations set the stage for potentially broad application in stereoselective lipase-catalyzed reactions, especially with the prospect of multiple recycling.

Enantioselective Reactions

It cannot be safely assumed that the result of an optimization procedure based on the model reaction involving the esterification of lauric acid (**1**) by *n*-octanol (**2**) with formation of the ester **3** also represents the best catalyst for the lipase-catalyzed transformation of other substrates. Thus, in a real industrial application involving a given substrate of interest, the first step should make use of the sol-gel catalysts already available, but in a second step it may be useful to perform the type of optimization described above. In the present case such an additional search was not performed. Rather, we simply tested some of the above optimized second generation sol-gel lipase immobilizates in the kinetic resolution of six different chiral substrates.

From an industrial point of view the quality of a given kinetic resolution not only depends upon the degree of enantioselectivity (which is the primary focus in academic publications), but also on the activity and the possibility of recycling and reusing the lipase (which are usually neglected in such investigations). We therefore studied all of these factors in a test reaction involving the acylating kinetic resolution of *rac*-2-octanol (**6**), although complete optimization was not strived for. All reactions were carried out on a small scale (0.5 mmol) and stereoselectivity was ascertained by measuring the selectivity factor E on the basis of the formula of Sih et al.[23] All kinetic resolutions were carried out in non-dried isooctane using vinyl acetate (**7**) as the acylating agent and CaLB, BcL and PfL as the lipases encapsulated in gels derived from iBTMS/TMOS (5:1).

Table 3 reveals some remarkable trends. Of the three lipases tested, CaLB is by far the most active and enantioselective. The commercial CaLB powder leads to a respectable E-value of 70 and a specific activity of

Table 2. Activity of sol-gel encapsulated lipases in the model reaction involving **1/2** compared to the use of the respective non-immobilized lipase powder. Reaction scale and conditions are the same as in Table 1. Amounts of lipase lyophilizates used in the sol-gel process at 3 mmol scale: PfL (150 mg; spec. activity = 91 μmol/min · g protein); BcL (150 mg; spec. activity = 193 μmol/min · g protein); MmL (150 mg; spec. activity = 236 μmol/min · g protein); AnL (150 mg; spec. activity = 12 μmol/min · g protein); CrL type VII (60 mg; spec. activity = 134 μmol/min · g protein); CrL (150 mg; spec. activity = 86 μmol/min · g protein); TIL (70 mg; spec. activity = 6 μmol/min · g protein); PpL (150 mg; spec. activity = 40 μmol/min · g protein); PrL (150 mg; spec. activity = 24 μmol/min · g protein).

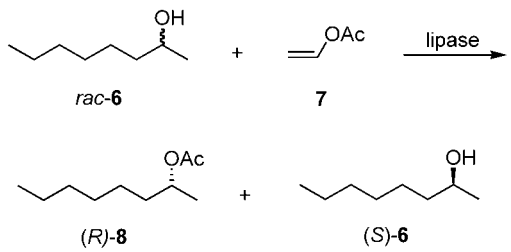
Entry	Lipase	Silane	Additives	Activity [μmol/min · g gel]	Specific Activity [μmol/min · g protein]	Relative Activity
1	PfL	**4d**	none	107	657	7.2
2	PfL	**4d**	Celite®[a]	123	1319	14.4
3	PfL	**4d**	18-crown-6[b]	119	1504	16.5
4	PfL	**4d**	Celite®[a]/18-crown-6[b]	97	1546	16.9
5	PfL	**4d**	methyl-β-CD[c]	127	883	9.7
6	PfL	**4d**	Celite®[a]/methyl-β-CD[c]	103	974	10.7
7	PfL	**4d**	Tween 80®[d]	192	1017	11.1
8	PfL	**4d**	Celite®[a]/Tween 80®[d]	105	846	9.3
9	PfL	**4e**	none	107	412	4.5
10	PfL	**4e**	Celite®[a]	60	621	6.8
11	PfL	**4e**	18-crown-6[b]	210	3275	35.8
12	PfL	**4e**	Celite®[a]/18-crown-6[b]	36	355	3.9
13	PfL	**4e**	methyl-β-CD[c]	207	1071	11.7
14	PfL	**4e**	Celite®[a]/methyl-β-CD[c]	83	739	8.1
15	PfL	**4e**	Tween 80®[d]	162	918	10.0
16	PfL	**4e**	Celite®[a]/Tween 80®[d]	46	348	3.8
17	BcL	**4d**	none	442	10771	55.7
18	BcL	**4d**	Celite®[a]	396	19775	102.0
19	BcL	**4d**	18-crown-6[e]	371	9280	48.0
20	BcL	**4d**	Celite®[a]/18-crown-6[e]	309	15435	79.8
21	BcL	**4d**	methyl-β-CD[f]	438	24333	126.0
22	BcL	**4d**	Celite®[a]/methyl-β-CD[f]	379	27100	140.0
23	BcL	**4d**	Tween 80®[g]	358	9421	48.7
24	BcL	**4d**	Celite®[a]/Tween 80®[g]	348	24864	129.0
25	BcL	**4e**	none	104	2088	10.8
26	BcL	**4e**	Celite®[a]	147	3984	20.6
27	BcL	**4e**	18-crown-6[e]	277	5896	30.5
28	BcL	**4e**	Celite®[a]/18-crown-6[e]	167	6958	36.0
29	BcL	**4e**	methyl-β-CD[f]	200	6663	34.5
30	BcL	**4e**	Celite®[a]/methyl-β-CD[f]	57	2850	14.7
31	BcL	**4e**	Tween 80®[g]	203	7526	38.9
32	BcL	**4e**	Celite®[a]/Tween 80®[g]	201	11161	57.7
33	MmL	**4d**	none	3224	7394	31.3
34	MmL	**4d**	Celite®[a]	1015	3679	15.6
35	MmL	**4e**	none	3953	6816	28.9
36	MmL	**4e**	Celite®[a]	655	2039	8.7
37	AnL	**4d**	none	34	129	11.1
38	AnL	**4d**	Celite®[a]	24	116	10.0
39	AnL	**4d**	Tween 80®[g]	34	114	9.8
40	AnL	**4d**	Celite®[a]/Tween 80®[g]	41	184	15.9
41	AnL	**4e**	none	22	50	4.3
42	AnL	**4e**	Celite®[a]	13	42	3.6
43	AnL	**4e**	Tween 80®[g]	28	76	6.6
44	AnL	**4e**	Celite®[a]/Tween 80®[g]	30	308	8.4
45	CrL (type VII)	**4d**	none	44	558	4.2
46	CrL (type VII)	**4d**	Celite®[a]	44	840	6.3
47	CrL (type VII)	**4e**	none	49	584	4.4
48	CrL (type VII)	**4e**	Celite®[a]	29	561	4.2
49	CrL (L-3)	**4d**	none	297	2122	24.6
50	CrL (L-3)	**4d**	Celite®[a]	58	226	2.6
51	CrL (L-3)	**4d**	Tween 80®[h]	245	1664	19.3
52	CrL (L-3)	**4d**	Celite®[a]/Tween 80®[h]	192	1861	21.6

Table 2 (cont.)

Entry	Lipase	Silane	Additives	Activity [μmol/min · g gel]	Specific Activity [μmol/min · g protein]	Relative Activity
53	CrL (L-3)	**4e**	none	256	1028	11.9
54	CrL (L-3)	**4e**	Celite®[a]	39	134	1.6
55	CrL (L-3)	**4e**	Tween 80®[h]	625	2225	25.8
56	CrL (L-3)	**4e**	Celite®[a]/Tween 80®[h]	40	232	2.7
57	TIL	**4d**	none	710	3947	617
58	TIL	**4d**	Celite®[a]	857	5909	923
59	TIL	**4d**	18-crown-6[i]	848	2968	465
60	TIL	**4d**	Celite®[a]/18-crown-6[i]	977	4862	760
61	TIL	**4d**	KCl[e]	646	2485	388
62	TIL	**4d**	Celite®[a]/KCl[e]	848	4816	753
63	TIL	**4d**	Tween 80®[j]	1208	6194	968
64	TIL	**4d**	Celite®[a]/Tween 80®[j]	1237	8899	1391
65	TIL	**4e**	none	405	1007	157
66	TIL	**4e**	Celite®[a]	577	2290	358
67	TIL	**4e**	18-crown-6[i]	742	2108	329
68	TIL	**4e**	Celite®[a]/18-crown-6[i]	899	3856	603
69	TIL	**4e**	KCl[e]	817	1901	297
70	TIL	**4e**	Celite®[a]/KCl[e]	947	2801	438
71	TIL	**4e**	Tween 80®[j]	644	2762	432
72	TIL	**4e**	Celite®[a]/Tween 80®[j]	823	4705	735
73	PpL	**4d**	none	126	302	7.5
74	PpL	**4d**	Celite®[a]	125	407	10.1
75	PpL	**4d**	Tween 80®[k]	55	134	3.3
76	PpL	**4d**	Celite®[a]/Tween 80®[k]	63	223	5.5
77	PpL	**4e**	none	40	67	1.7
78	PpL	**4e**	Celite®[a]	47	122	3.0
79	PpL	**4e**	Tween 80®[k]	142	257	6.4
80	PpL	**4e**	Celite®[a]/Tween 80®[k]	35	88	2.2
81	PrL	**4d**	none	15	78	3.2
82	PrL	**4d**	Celite®[a]	10	74	3.1
83	PrL	**4d**	18-crown-6[l]	8	50	2.1
84	PrL	**4d**	Celite®[a]/18-crown-6[l]	10	94	3.9
85	PrL	**4e**	none	16	57	2.4
86	PrL	**4e**	Celite®[a]	8	47	2.0
87	PrL	**4e**	18-crown-6[l]	74	640	26.7
88	PrL	**4e**	Celite®[a]/18-crown-6[l]	9	56	2.3

[a] 50 mg. [b] 1 mmol. [c] 140 mg. [d] 30 mg. [e] 2 mmol. [f] 40 mg. [g] 60 mg. [h] 180 mg. [i] 0.5 mmol. [j] 120 mg. [k] 90 mg. [l] 1.5 mmol.

188 μmol min^{-1} g^{-1} protein, but upon immobilization at high loading in the absence of an additional additive these increase to $E > 100$[24] and 790 μmol min^{-1} g^{-1} protein, respectively (Table 3, entry 1/2). Thus, lipase-entrapment causes a significant enhancement in enantioselectivity, while also increasing the activity by a factor of 4.2. The additive 18-crown-6 has only a small effect (entry 3). In the case of the other two lipases (BcL and PfL) the substrate shows poor enantioselectivity irrespective of immobilization, although rate enhancement is observed.



Scheme 3.

The CaLB-immobilizate was then subjected to a series of recycling experiments. The gel was separated, washed and reused, the total number of reactions amounting to 20. The selectivity factor remained constant at $E > 100$ over all cycles. Moreover, at least 70% of enzyme activity is retained after 20 reactions, whereas only 40% is present when using the commercial CaLB powder (Figure 3).

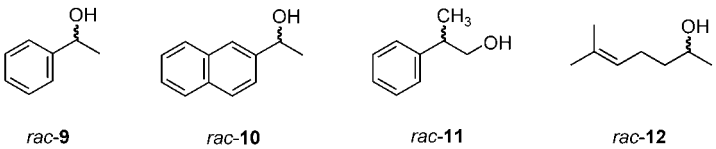
Table 3. Kinetic resolution of alcohol **6** using free and sol-gel encapsulated lipases (Table 1) as catalysts, vinyl acetate as the acylating agent and isooctane as solvent.

Entry	Lipase	Additives used in Sol-Gel Process	Activity [μmol/min · g gel]	Specific Activity [μmol/min · g protein]	Relative Activity	E
1	CaLB (powder)	none	100	188	–	70.1
2	CaLB (sol-gel)	none	529	790	4.2	>100
3	CaLB (sol-gel)	18-crown-6	425	966	5.1	>100
4	BcL (powder)	none	6	51	–	4.4
5	BcL (sol-gel)	none	14	288	5.6	4.1
6	BcL (sol-gel)	18-crown-6	45	964	18.8	4.0
7	BcL (sol-gel)	Tween 80®	28	937	18.3	4.5
8	PfL (powder)	none	6	34	–	2.2
9	PfL (sol-gel)	none	28	108	3.2	3.3
10	PfL (sol-gel)	18-crown-6	40	625	18.4	6.0

Similar (non-optimized) experiments were then performed with the chiral alcohols **9** – **12**. In almost all cases sol-gel entrapment has a strongly beneficial effect as measured in the first cycle (Table 4). An exception is the kinetic resolution of 1-phenylethanol (*rac*-**9**) catalyzed by CaLB. Although the selectivity factor ($E > 100$) is not effected by sol-gel encapsulation, the lipase activity is reduced slightly (Table 4), which is currently difficult to explain. Of course, the advantage regarding the possibility of recycling the heterogeneous catalyst remains. In several other cases involving other substrates recycling experiments were also performed, again illustrating industrial viability. These as well as our original recycling experiments[10,11] involving the first generation immobilizates show that sol-gel encapsulated lipases are extremely robust heterogeneous biocatalysts.

A disadvantage of using enzymes in organic synthesis is the fact that in many cases the amount of biocatalyst used weight-wise equals or even exceeds the weight of substrate used in the reaction.[25] In the case of the present immobilizates this is not the case, in spite of the fact that the major part of the catalyst weight-wise originates from the silicate matrix. For example, in a preparative scale reaction of *rac*-**10**, only 250 mg of sol-gel CaLB-immobilizate was used per 10 g of substrate (see Experimental Section).

Finally, the acylating kinetic resolution of 1-phenylethylamine (*rac*-**13**) was studied. Whereas the reaction is extremely slow when employing ethyl acetate as the acylating agent (reaction time: 7 days),[26] scientists at BASF discovered that methoxyacetic acid ethyl ester (**14**) results in dramatic rate acceleration.[27] Upon using the lipase from *Burkholderia plantarii*, the (*R*)-amide (**15**) was obtained having an enantiomeric purity of ee = 93% at 52% conversion, while the (*S*)-amine (**13**) was isolated enantiomerically pure (ee = 99%).[27] The *E*-value was reported to be > 100. Upon immobilizing the



rac-**9** *rac*-**10** *rac*-**11** *rac*-**12**

Scheme 4.

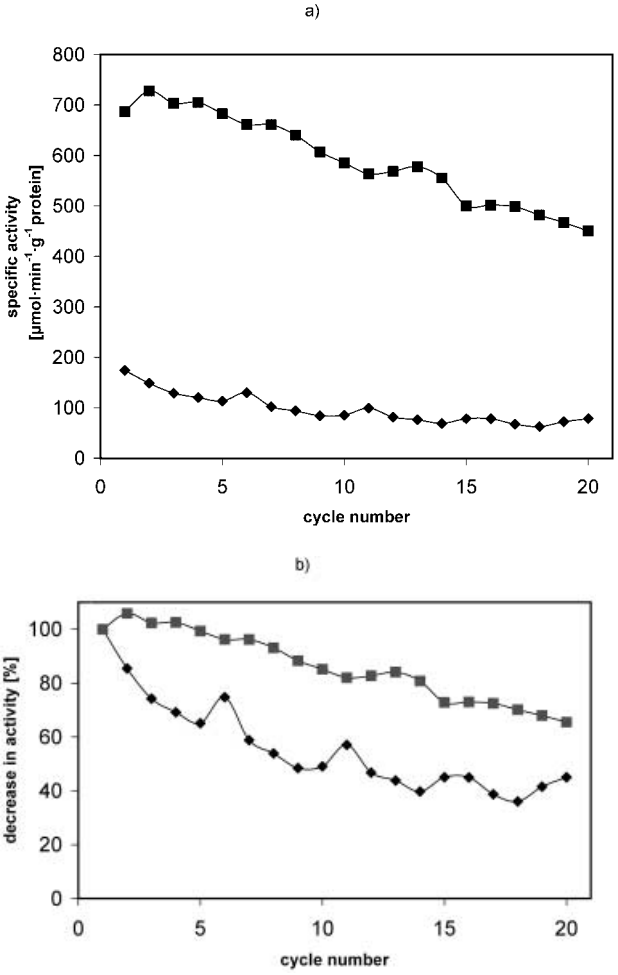


Figure 3. Recycling experiments in the kinetic resolution of *rac*-**6** using sol-gel CaLB-immobilizate (–■–) and commercial CaLB powder (–◆–). a) Specific activity. b) Decrease in activity (%).

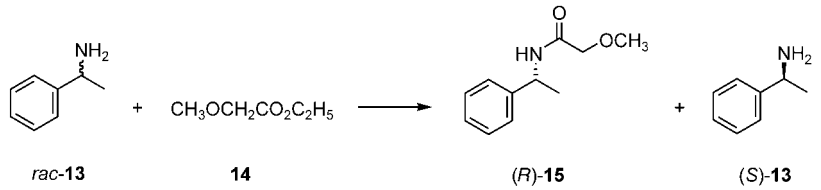
Table 4. Kinetic resolution of alcohols **9** – **11** using free and sol-gel encapsulated lipases (Table 1) as catalysts, vinyl acetate as the acylating agent and isooctane as solvent.

Entry	Substrate	Lipase	Additives	Activity [μmol/min · g gel]	Specific Activity [μmol/min · g protein]	Relative Activity	E
1	**9**	CaLB (powder)	none	467	876	–	>100
2	**9**	CaLB (sol-gel)	none	292	436	0.5	>100
3	**9**	CaLB (sol-gel)	18-crown-6	332	755	0.9	>100
4	**9**	BcL (powder)	none	8	63	–	>100
5	**9**	BcL (sol-gel)	none	35	700	11.1	>100
6	**9**	BcL (sol-gel)	18-crown-6	50	1057	16.8	>100
7	**9**	BcL (sol-gel)	Tween 80®	42	1393	22.2	>100
8	**9**	PfL (powder)	none	3	15	–	>100
9	**9**	PfL (sol-gel)	none	21	80	5.3	>100
10	**9**	PfL (sol-gel)	18-crown-6	25	392	26.1	>100
11	**10**[a]	CaLB (powder)	none	214	401	–	>100
12	**10**[a]	CaLB (sol-gel)	none	214	320	0.8	>100
13	**10**[a]	CaLB (sol-gel)	18-crown-6	253	575	1.4	>100
14	**10**[a]	BcL (powder)	none	6	51	–	>100
15	**10**[a]	BcL (sol-gel)	none	59	1174	22.9	>100
16	**10**[a]	BcL (sol-gel)	18-crown-6	36	774	15.1	>100
17	**10**[a]	BcL (sol-gel)	Tween 80®	23	777	15.2	>100
18	**10**[a]	PfL (powder)	none	6	32	–	>100
19	**10**[a]	PfL (sol-gel)	none	23	87	2.8	>100
20	**10**[a]	PfL (sol-gel)	18-crown-6	14	225	7.1	>100
21	**11**	CaLB (powder)	none	920	1726	–	1.9
22	**11**	CaLB (sol-gel)	none	444	664	0.4	2.0
23	**11**	CaLB (sol-gel)	18-crown-6	746	1695	1.0	1.2
24	**11**	BcL (powder)	none	51	419	–	1.1
25	**11**	BcL (sol-gel)	none	98	1956	4.7	2.7
26	**11**	BcL (sol-gel)	18-crown-6	219	4662	11.1	2.5
27	**11**	BcL (sol-gel)	Tween 80®	145	4843	11.6	2.4
28	**11**	PfL (powder)	none	68	367	–	1.2
29	**11**	PfL (sol-gel)	none	186	713	1.9	1.6
30	**11**	PfL (sol-gel)	18-crown-6	84	1311	3.6	1.5
31	**12**[b]	BcL (powder)	none	0.040	0.33	–	29.1
32	**12**[b]	BcL (sol-gel)	none	0.262	5.2	15.9	31.6
33	**12**[b]	BcL (sol-gel)	18-crown-6	0.262	5.6	16.9	40.2
34	**12**[b]	BcL (sol-gel)	Tween 80®	0.323	12.0	36.2	32.6

[a] Solvent: toluene. [b] Solvent: tetrahydrofuran.

lipase on Amberlite XAD7 and carrying out the reaction 10 times, the remaining activity was measured to be 80%. The BASF plant has started to produce the amine at a scale of 1000 tons/year.[28]

Since the lipase from *Burkholderia plantarii* is not commercially available, we began to test the lipases accessible in the present study. Since we already knew that CaLB is very enantioselective in the acylation of **13** using ethyl acetate,[26] the reaction was repeated with **14** as the acylating agent. It was observed that the commercial powder as well as the sol-gel (from **4e**/**5**) encapsulated forms with or without 18-crown-6 are excellent catalysts, the desired 50% conversion being reached within 29 hours and the enantioselectivity being very high ($E > 100$). This means that the reaction essentially stops when the (*R*)-enantiomer of **13** has been consumed, which is ideal. Of the three catalysts studied, the sol-gel encapsulated variant prepared in the presence of 18-crown-6 turned out to be most active. Therefore, recycling experiments were performed with



rac-**13** **14** (*R*)-**15** (*S*)-**13**

Scheme 5.

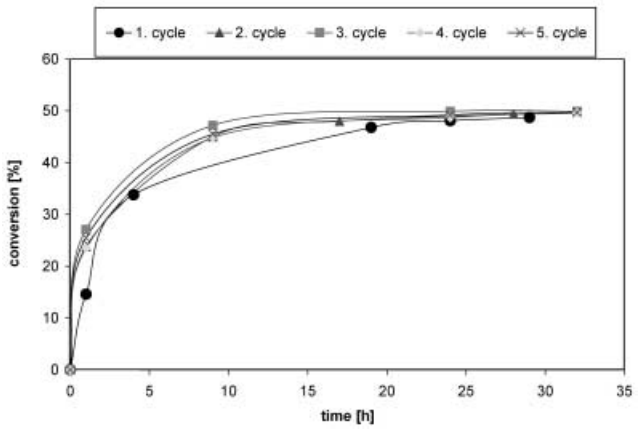


Figure 4. Recycling experiments in the kinetic resolution of *rac*-**13** using the sol-gel CaLB-immobilizate prepared with 18-crown-6 as an additive.

this material. Figure 4 shows the results of five cycles, which demonstrate constant performance with respect to activity. Moreover, no reduction in enantioselectivity was observed in any of the runs.[22a]

Conclusions

The present study reveals that the second generation sol-gel lipase immobilizates are extremely active, robust and recyclable heterogeneous biocatalysts in esterification reactions. The preparation involves high lipase loading as well as the use of additives such as 18-crown-6 or Tween 80®. Ten different lipases were tested, and all of them showed significantly improved performance, although in two cases additive effects were not observed. Upon going from *n*-butyl- to isobutyltrimethoxysilane (**4d** and **4e**, respectively) as the precursor in the sol-gel process, differences in activity and enantioselectivity are usually small, although notable exceptions were observed depending upon the particular substrate used. In typical preparative scale reactions only about 250 mg of a lipase-containing gel are needed for 10 g of substrate.

In future applications involving new substrates it is advisable to test several variants of the sol-gel encapsulated lipases described herein. In industrial applications this should include those materials prepared by "double immobilization" based on the use of an additional solid porous material such as Celite® which bonds in its pores the actual lipase-containing gels and imparts enhanced mechanical stability as well as activity. Future studies in our laboratories will focus on the origin of improved catalyst performance.

Experimental Section

Materials and Instruments

The lipases from *Candida antarctica* (CaLB; Chirazyme L-2®), *Candida rugosa* (CrL; Chirazyme L-3®) and *Mucor miehei* (MmL; ChirazymeL-9®) were obtained from Roche; *Aspergillus niger* (AnL; Amano AS), *Burkholderia cepacia* (BcL; Amano PS) and *Pseudomonas fluorescens* (PfL; Amano AK "20") from Amano Pharmaceutical Co.; *Candida rugosa* type VII (CrL type VII) from Sigma; *Penicillium roqueforti* (PrL) from Fluka; *Thermomyces lanuginosa* (TIL; Novozym SP523) from Novo Nordisk. The chiral alcohols were purchased from Aldrich; isobutyltrimethoxysilane (98%) from Lancaster; *n*-butyltrimethoxysilane (97%) from ABCR; Tween 80® [polyoxyethylene(20)sorbitan monooleate] from Fluka; polyvinyl alcohol (PVA; MW =15000) from Merck; sodium fluoride (99%) from Fluka; vinyl acetate (99%) from Acros; Celite®577 from Fluka; isooctane (99%) from Fluka; methyl-β-cyclodextrin from Aldrich; 18-crown-6 (99%) from Fluka; tetramethoxysilane (98%) from Fluka; lauric acid (99%) from Fluka; 1-phenylethylamine (98%) from Fluka; methoxyacetic acid ethyl ester (97%) and *n*-octanol (99.5%) from Fluka.

Gas chromatographic analyses were performed in the Department of Chromatography, Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr, Germany.

General Procedure for Sol-Gel Entrapment of Lipases

A commercial lipase powder (lyophilizate) such as AnL (150 mg), BcL (150 mg), CaLB (125 mg), CrL (150 mg), CrL type VII (60 mg), MmL (150 mg), PfL (150 mg), PpL (150 mg), PrL (150 mg) or TIL (70 mg) was placed in a 50-mL Falcon tube (Corning) together with TRIS/HCl-buffer (390 μL; 0.1 M; pH 7.5) and the mixture was vigorously shaken with a Vortex-Mixer. In the case of an additive or Celite®, these materials were included, the amounts of which are listed in the tables. Then 100 μL of aqueous PVA (4% W/V), aqueous sodium fluoride (50 μL of a 1 M solution) and isopropyl alcohol (100 μL) were added, and the mixture homogenized using a Vortex-Mixer. Then the alkylsilane (2.5 mmol) and TMOS (0.5 mmol; 74 μL; 76 mg) were added and the mixture agitated once more for 10 to 15 seconds. Gelation was usually observed within seconds or minutes while gently shaking the reaction vessel. Following drying overnight in the opened Falcon tube, isopropyl alcohol (10 – 15 mL) was added in order to facilitate removal of the white solid material (filtration). The gel was successively washed with distilled water (10 mL), isopropyl alcohol (10 mL) and *n*-pentane (10 mL). During this process a spatula was used to crush the gel. Thereafter the lipase immobilizate was placed in an open 2-mL plastic vessel and dried in the air at room temperature.

Determination of Protein Content

In order to determine the protein content of the commercial lipases as well as the degree of loading, the *BCA Protein Assay Kit* of Sigma was used. Accordingly, solutions of the commercial lipase or the wash-solutions following the sol-gel process were incubated for 30 min and measured at 37 °C using a UV/

Vis-spectrometer at 562 nm according to the *Technical Bulletin* of Sigma. Distilled water was used as a reference and BSA as standards. The degree of immobilization ranges between 0.3 and 0.9.

Determination of Lipase Activity

The sol-gel lipase immobilizate (1 to 50 mg, depending on the activity) was placed in a Falcon tube together with a solution of lauric acid (100 mg; 0.5 mmol) and *n*-octanol (158 μL; 130 mg; 1.0 mmol) in non-dried (H_2O-saturated) isooctane. The mixture was then shaken (180 min^{-1}) at 30 °C. At defined intervals (usually after 15, 30, 45 and 60 min) samples (300 μL) were taken and centrifuged (13000 min^{-1}) before the gas chromatographic determination of lauric acid and lauric acid *n*-octyl ester was carried out using 150 μL of the supernatant. By applying a linear regression of the measured values the initial reaction rate in μmol/min and therefore the activity relative to 1 g of immobilizate can be determined. By considering the degree of loading, the specific activity is calculated. The relative activity is determined by dividing the specific activity of the immobilizate by the specific activity of the commercial lipase powder.

General Procedure for Evaluating Lipase-Immobilizates as Catalysts in the Kinetic Resolution of Chiral Alcohols

A Falcon tube (Corning) was charged with a mixture of a racemic alcohol (0.5 mmol), vinyl acetate (93 μL; 86 mg; 1 mmol) and free or sol-gel immobilized lipase [typically 5 mg in 10 mL solvent (typically isooctane)]. The mixture was shaken at 30 °C, samples (150 μL) being taken for GC analysis at 15 minutes intervals. The formula of Sih et al.[23] was used to calculate the *E*-value. For recycling experiments the reaction mixture was centrifuged, the supernatant was decanted and the lipase or the sol-gel immobilizate washed 4 times with isooctane and reused without any further treatment.

Typical Kinetic Resolution on a Preparative Scale

Similar to the above protocol a mixture of *rac*-1-(2-naphthyl)-ethanol (10 g; 58 mmol), vinyl acetate (8.1 mL; 7.5 g; 87 mmol) and 250 mg of a sol-gel CaLB-immobilizate (prepared in the presence of 18-crown-6 as additive) in toluene (300 mL) was shaken at 35 °C for 48 h. Following filtration GC analysis showed a conversion of 50.0%, the ee of non-reacted (*S*)-1-(2-naphthyl)ethanol and product ((*R*)-acetate) each being > 99.9%. The immobilizate was removed by filtration, washed with toluene and pentane and can be reused without significant loss in activity or enantioselectivity.

General Procedure for the Kinetic Resolution of 1-Phenylethylamine

rac-1-Phenylethylamine (127.4 μL; 121.3 mg; 1.0 mmol), methoxyacetic acid ethyl ester (352.2 μL; 354.3 mg; 3.0 mmol) and 75 mg of free or immobilized lipase B from *Candida antarctica* were added to H_2O-saturated toluene (10 mL). The reaction mixture was shaken at ambient temperature (230 min^{-1}) for several hours. After defined intervals samples of 50 μL were taken, after which they were diluted with 400 μL toluene and centrifuged for 2 min (13000 min^{-1}). 200 μL of the supernatant were analyzed via gas chromatography. For recycling of the immobilizates the reaction mixture was filtered and the immobilizates were washed three times with 10 – 15 mL toluene and once with 10 – 15 mL pentane. After drying the entrapped lipase for 24 h at room temperature under air, they were reused in the kinetic resolution described above. For this reason the amounts of reactants were adjusted to the amount of recycled immobilizate.

Acknowledgements

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