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Lipases from *Candida antarctica*: Unique Biocatalysts from a Unique Origin

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Abstract:

The specificity of the A-lipase from *Candida antarctica* (CALA) has been characterized to further clarify the scope of the biocatalyst. The lipase was found to exhibit an almost uniform activity towards various straight-chained primary alcohols and carboxylic acids, only exhibiting a low activity towards very short-chained acids. More interestingly, the enzyme was found to exhibit a high activity towards a surprising diversity of sterically hindered alcohols, including both secondary and tertiary alcohols. These results indicate that CALA can have a unique applicability for the conversion of highly branched substrates where most other lipases fail to display any activity. A new, potentially highly cost-effective, immobilization technology using silica-based granulation has been applied in the immobilization of the B-lipase from the same yeast (CALB). Highly stable particles were obtained with an activity comparable to that of the commercially available immobilized preparations of this enzyme.

Introduction

The basidiomyceteous yeast *Candida antarctica* produces two different lipases, named A and B.[1,2] Throughout this contribution, CALA and CALB will be used as abbreviations for the A- and B-lipase, respectively. As the name suggests, the strain was originally isolated in Antarctica, with the aim of finding enzymes with extreme properties, and both CALA and CALB do, indeed, exhibit unusual properties. As indicated in Table 1 the two lipases are very different.[3−5] The stability of any enzyme is a critical factor determining its applicability. Both these lipases are stable over a relatively broad pH range with CALA being most stable at acidic pH and CALB being most stable at alkaline pH. CALA is an extremely thermostable protein, and the enzyme has its temperature optimum above 90 °C.[6] The lipase is, in fact, probably the most thermostable lipase described to date, a surprising feature given its origin from a cold-adapted organism. Even though CALB is not as stable as CALA in solution, both lipases are highly stable in an immobilized form and can be used in operation at elevated temperature for thousands of hours without any significant loss in activity.[7] Activity and specificity are, of course, other critical factors defining the usability of an enzyme catalyst. As outlined below, perhaps the most unique features of CALA and CALB are their substrate specificities that in many applications have been found to be very different from most other known lipases, thereby opening up for unique applications.

Table 1. **Characteristics of CALA and CALB**

	CALA	CALB
molecular weight (kD)[3]	45	33
isoelectric point (pI)[3]	7.5	6.0
pH optimum[4]	7	7
specific activity (LU/mg)[4]	420	435
thermostability at 70 °C[3,a]	100 [100]	15 [0]
pH stability[3,b]	6−9	7−10
interfacial activation[4]	yes (but low)	No
positional specificity toward triglycerides[5]	*Sn*-2	*Sn*-3

[a] Residual activity after incubation at 60 °C in 0.1 M tris buffer (pH 7.0) for 20 min and [120 min]. [b] pH at which more than 75% activity is retained following incubation for 20 h at room temperature.

Activity and Specificity of CALA. Relatively few studies have been published on this lipase to date. Initial investigations did not reveal any unusual specificity of the enzyme. Even though it has a preference for the *sn*-2 position in triglycerides, this selectivity is not pronounced enough to enable selective synthesis of 1,3-diglycerides or 2-monoglycerides. In practical interesterification of triglycerides, the lipase is rather nonselective.[7] Furthermore, early work on the lipase did not reveal any unusual specificity in the esterification of simple alcohols[7] and as it, moreover, was found to have a relatively low specific activity compared to many other lipases, there were no indications that the enzyme possessed any unique features apart from the extremely high thermostability. This feature did, however, open up for some applications in which operation in solution at very high temperature was required. Thus, the enzyme has found use in the paper and pulp and textile industries where it is used to hydrolyze very high-melting fatty acid glycerides and resin esters.[8] Some more recent studies focusing on organic synthesis have, however, indicated that the enzyme can exhibit a highly unique specificity towards some selected substrates. CALA has been tested among other lipases in

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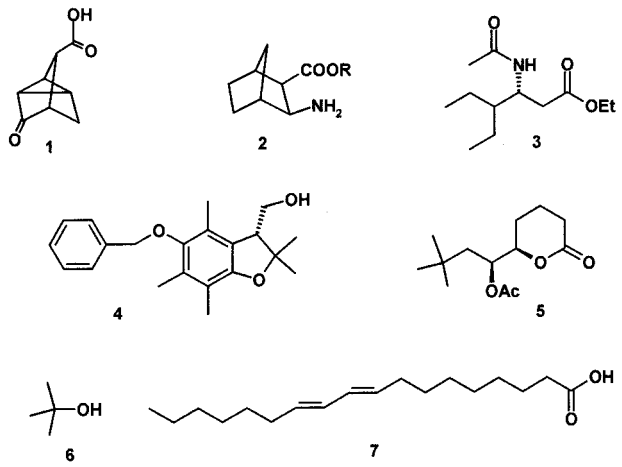

Figure 1. **Examples of unique substrates accepted by CALA.**

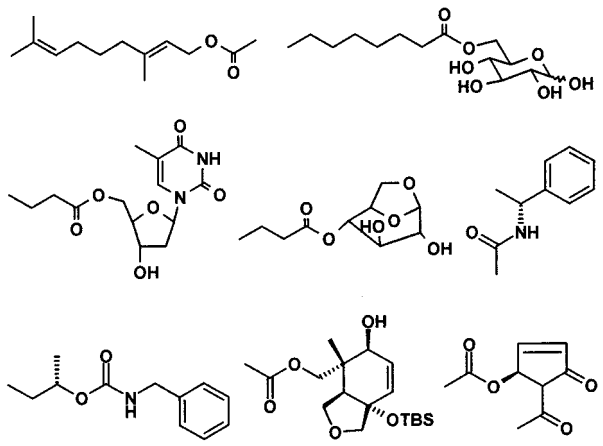

Figure 2. **Examples of substrates successfully synthesized using CALB as catalyst.[19]**

the search for enantioselective catalysts in several studies, but has in most cases failed to provide any interesting results. However, in the esterification of highly sterically hindered substrates, CALA has in several studies been found to be the only lipase able to catalyze the desired reactions. The enzyme has been found to be able to accept highly branched acyl groups as well as sterically hindered alcohols and amines. Some examples of substrates accepted by CALA are shown in Figure 1. Towards these substrates, CALA exhibits a highly useful selectivity. Thus, in the preparation of **1**, **2**, **3**, and **4** CALA-catalyzed synthesis provided an enantiomeric excess of 99,[9,10] 99,[11] 98,[12] and 87%,[13] respectively, and in the preparation of **5**, CALA exhibited a *Z*/*E* selectivity of 100/0 at 94% conversion.[14] CALA has in the patent literature been claimed to be able to esterify even tertiary alcohols such as **6**.[15] This is a very unique feature as tertiary alcohols, such as *t*-butyl alcohol, are often used as more polar solvent when this is required in reactions using other lipases as catalysts as these enzymes exhibit no activity at all towards tertiary alcohols. Finally, also in the esterification of *cis/trans*-isomers of unsaturated fatty acids such as **7**, CALA has shown a unique selectivity. While other lipases tend to favor the esterification of *cis*-fatty acids, CALA has in several studies been found to have a clear preference for the *trans*-isomer.[16−18]

In summary, while early studies did not indicate that CALA possessed any unique specificity, more recent studies have, indeed, indicated that this is not the case. First of all, it seems that CALA may have a unique ability to accept very bulky substrates. Even though several unique applications of CALA, accordingly, have been identified, the potential of the enzyme is, most likely, still far from being fully explored. In this report we present a more fundamental characterization of the selectivity of CALA that can, hopefully, further help identifying new applications for this unique catalyst.

Activity and Specificity of CALB. In contrast to CALA, CALB is a very well characterized catalyst and its highly diversified use has recently been reviewed.[19] In immobilized form, the catalyst has been found to tolerate a great variation in experimental conditions and it has in numerous publications been shown to be a particularly efficient enzyme catalyzing a great number of different organic reactions including many that have been scaled up to commercial scale.

CALB exhibits a very high degree of substrate selectivity both with respect to regio-selectivity and enantioselectivity. CALB has been used intensively as a region-selective catalyst, first of all to selectively acylate different carbohydrates.[19] For CALB, however, the most extensive area of use is in the resolution of racemic alcohols, amines, and acids, or the preparation of optically active compounds from *meso* reactants. The resulting optically pure compounds are highly difficult to obtain by alternative routes and can be of great synthetic value. The diversity of products successfully synthesized using CALB as a regio- or enantioselective catalyst is illustrated in Figure 2.

CALB has a very broad substrate specificity,[20] and the lipase can potentially be used in a number of other synthetic reactions, leading to the formation of less complex structures. The primary factor limiting the present industrial use of CALB is, most likely, the relatively high price of the immobilized products available today, Novozym 435 (Novozymes A/S) and Chirazyme L-2 (Roche Molecular Biochemicals). These products are primarily targeted for the resolution of chiral intermediates and other high-priced specialty chemicals where performance and purity of the catalyst is more critical than the price. If a less expensive immobilized preparation could be made available, it could,

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most likely, open up for new applications focusing on more commodity-type products such as aliphatic polyesters, biodiesel, and other simple esters used in bulk quantities.

Lipase Immobilization. Immobilization technology is a key technology for the applied usages of biocatalysts, which ensure that the biocatalyst can easily be removed from reaction mixture and, preferably, be reused. Furthermore, immobilization does also, in general, increase the thermostability of the enzyme by stabilizing the tertiary structure of the protein. Several immobilization technologies can be used for the immobilization of lipases,[21−23] for example: (1) adsorption onto polymer based carriers, (2) covalent linkage to carriers, for example, epoxy-based chemistry, (3) cross-linking of enzyme protein using, for example, glutaraldehyde, 4) encapsulation (e.g., in alginate beads or siloxane matrices), and (5) deposition onto hydrophilic inorganic material, for example, Celite types.

From a large-scale production standpoint several factors become important when considering possible immobilization technologies: (1) The procedures should preferably be robust and re-producible; (2) the production logistics should entail no process steps with a high-holding time to ensure a maximum capacity; (3) enzyme stability during each process steps has to be robust; (4) handling of, for example, cross-linking chemicals and dust-producing materials should be taken into account; and (5) correlation with pilot-scale immobilization will be preferable when considering future process developments. In this report we present a new approach for immobilizing CALB based on silica granulation, a technology addressing most of the above factors, and the characterization of these products.

Results and Discussion

Substrate Specificity of CALA. For the use in the specificity studies CALA was immobilized onto porous polypropylene following standard procedures.[24] The specificity of CALA towards various straight-chain carboxylic acids has previously been investigated. The observation previously reported that CALA tends to prefer more long-chained straight-chained carboxylic acids[7] was confirmed using CALA immobilized on polypropylene as no reaction was observed with acetic and butyric acid, while the highest reaction rates were achieved using more long-chained carboxylic acids (results not shown). As the specificity of CALA towards highly branched carboxylic acids and towards various unsaturated acids, as indicated above, is well investigated, the studies were concentrated on the activity of the enzyme towards various alcohols.

Initially, the specificity of CALA towards primary, straight-chained aliphatic alcohols of varying chain length was investigated. Thus, specificity constants ($1/\alpha$) were determined for each alcohol in a series of competition experiments performed using 1-hexanol as reference (Table 2) and dodecanoic acid as acyl donor. The specificity constants indicate the enzyme to exhibit an almost identical preference for all the lower alcohols, while a lower preference for linear primary alcohols with more than 10 carbon atoms. Interestingly, the enzyme also seems to have a preference for the shortest alcohol tested, methanol.

Table 2. **Specificity of CALA towards straight-chained primary alcohols relative to 1-hexanol using dodecanoic acid as acyl donor (100 mg of immobilized CALA, 6 mL of reaction mixture, 0.2 M acid and 0.2 M total alcohol, room temperature, speed of stirring 30 rpm)**

substrate	competitive factor, α	specificity constant, $1/\alpha$
methanol	1.23	0.81
ethanol	1.03	0.97
1-propanol	1.00	1.00
1-butanol	0.92	1.08
1-pentanol	0.97	1.03
1-hexanol	1	1
1-octanol	0.93	1.08
1-decanol	0.74	1.35
1-dodecanol	0.74	1.35
1-hexadecanol	0.54	1.87
1-octadecanol	0.52	1.91

Table 3. **Activity of CALA towards various substituted alcohols using dodecanoic acid as acyl donor (100 mg of immobilized CALA, 6 mL of reaction mixture, 0.2 M acid and 0.2 M alcohol, room temperature, speed of stirring 30 rpm)**

substrate	initial rate (μmol/mg/min)
1-hexanol	0.120
benzyl alohol	0.082
2-phenyl-ethanol	0.198
2-phenyl-1-propanol	0.222
2-ethyl-1-hexanol	0.111
2-propanol	0.031
2-butanol	0.056
R,*S*-2-octanol	0.052
S-2-octanol	0.054
3-pentanol	0.082
2-methyl-2-propanol (*tert*-butyl alcohol)	0.073
2-methyl-2-butanol (*tert*-pentyl alcohol)	0.017
cholesterol	0.075
β-sitosterol	0.113

The activity of the enzyme towards various substituted primary alcohols as well as secondary and tertiary alcohols was then investigated. In this study initial rates were measured in individual experiments performed, again using dodecanoic acid as acyl donor and 1-hexanol as reference. As indicated in Table 3, CALA exhibited a surprisingly high activity towards all the sterically hindered alcohols tested as initial rates comparable to the one obtained using 1-hexanol as substrate was observed in all experiments. These results further confirm the indications in the literature that CALA has a unique activity towards more bulky substrates The lipase exhibited even an almost 2-fold higher activity towards phenyl-substituted alcohols with the phenyl group in the 2-position, while a somewhat lower activity was seen

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using benzyl alcohol as substrate. The reports in the patent literature[15] that CALA is capable of esterifying tertiary alcohol was confirmed because an excellent activity was observed towards *tert*-butyl alcohol, while a somewhat lower activity was observed using *tert*-pentyl alcohol as substrate. To the best of our knowledge no other lipase has to date been reported as being able to accept tertiary alcohols as substrate. Finally, the poor enantioselectivities observed in several previous studies using various simple secondary alcohols as substrates were confirmed as almost identical initial rates were observed in experiments using racemic 2-octanol and the pure *S*-enantiomer as substrates.

Esters of various phytosterols are interesting products as they have been reported to exhibit beneficial nutritional effects when added to fat blends such as margarine.[25] As these alcohols are highly sterically hindered, they are poor substrates of most other lipases tested. As CALA is able to esterify other sterically hindered alcohols, the activity of the enzyme was tested towards the cholesterol and β-sitosterol alcohols. As indicated in Table 3, CALA was able to esterify both steroid alcohols with an activity comparable to most other alcohols tested.

Immobilization of CALB by Silica Granulation. Granulation is an important formulation technology for both pharmaceutical applications as well as for enzyme applications. A well-known example for the latter application is enzyme granules for detergent powder where the enzyme is released into the washing liquor during the washing. Immobilization onto inorganic type of carriers or powder has been reported to a great extent. Using granulation technology to build up the carrier with lipase adsorbed on silica powder is, on the contrary, a new development within immobilization technology.[26,27] Due to the composition of the granulates, they are intended for micro-aqueous mixtures only. Lipase will desorb and the particle will slowly disintegrate if taken into aqueous systems. However, the CALB silica granules are applicable in a direct ester synthesis reaction where water is formed, provided that the formed water is removed from the reaction mixture. Either vacuum, azeotrope distillation, or prevaporization can be applied to remove water produced. This also helps forcing the reaction to completion. Applying the granules in packed bed reactors also minimizes the contact time with higher water concentrations.

The physical characteristics of the pilot plant preparations (NS 40013) were characterized with respect to particle size distribution, surface area measurement, and bulk density. A series of granulations were performed by varying the lipase activity dosage (5, 10, 15, 20, and 41 kLU/g of dry matter of total granulation mixture, respectively). The physical yield in those experiments was in the range 68−78% of the theoretical yield. The obtained particle size distribution, measured by sieve analysis, is shown in Figure 3. Table 4 summarizes the physical characteristics of the silica granules.

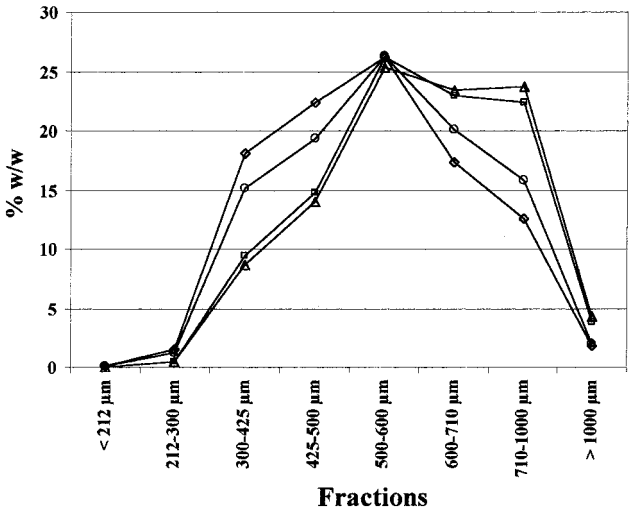

Figure 3. **Particle size distribution of CALB granulates. (△) NS 40013 5 kLU CALB/g. (□) NS 40013 10 kLU CALB/g. (○) NS 40013 15 kLU CALB/g. (◇) NS 40013 20 kLU CALB/g. Data not shown for NS 40013, 41 kLU CALB/g.**

Table 4. **Physical characteristics of CALB granulate compared to Novozym 435**

preparation	surface area (m^2/g)	bulk density (g/mL)	porosity (mL/g)
NS 40013, 20 kLU/g	48−55	0.58	0.72
Novozym 435	80	0.42	0.34

We were able to produce silica granules with a particle size distribution comparable to many other polymer-based enzyme carriers and with a medium surface area (48−55 m^2/g). However, the porosity of NS 40013 20 kLU CALB/g (determined as bed porosity) was high (0.72) compared to that of Novozym 435 (0.34). As for normal heterogeneous catalysis, surface areas as well as porosity and particle size are very important factors when assessing the catalytic performance of, in this case, an immobilized biocatalyst. The granules obtained were incompressible, exhibited minimum swelling characteristics and were mechanically stable and therefore suitable for applications in packed bed reactors as well as stirred batch operations.

Performance of Silica-Granulated CALB in Ester Synthesis. The catalytic activity performance of the granulates were compared to Novozym 435 in an industrial relevant application, the synthesis of *iso*-propyl myristate, an oleochemical used as a skin emollient. Two different silica preparations were tested (lipase dosage 20 and 41 kLU/g respectively, sieve fraction 300−1000 μm) and the time course of the reaction is shown in Figure 4. The estimated relative initial activities were calculated to: Novozym 435 (normalized to 100%), NS 40013 batch 41 kLU CALB/g (47%) and NS 40013 batch 20 kLU CALB/g (22%). In this particular case, we observed approximately 100% increase in initial activity when applying double the amount of lipase in the granulation. However, we do not expect to find a linear correlation between lipase dosage in the granulation and the observed initial activity in future preparations. Since the lipase is first adsorbed onto the silica powder and then agglomerated, lipase protein does occur throughout the core

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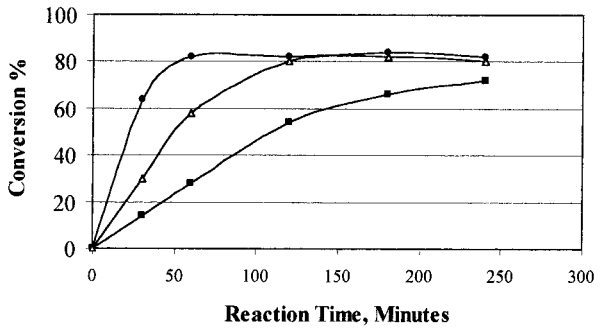

Figure 4. **CALB-catalyzed synthesis of *iso*-propyl myristate (solvent-free reaction: 21 mmol acid dissolved in 83 mmol alcohol, 10% catalyst (w/w), 40 °C, speed of stirring 150 rpm). (■) NS 40013 20 kLU CALB/g. (△) NS 40013 41 kLU CALB/g. (●) Novozym 435.**

of the particle and not only on the surface of it. The apparent activity is therefore dependent not only on the amount of adsorbed lipase but also on the physical access to the lipase protein, in terms of pore size, surface area, and pore volume in connection with mass transfer and film diffusion problems.

Conclusions

It is well established that both CALA and CALB are highly unique lipases. Even though both enzymes have found various uses their full potential is, most likely, far from being fully explored. The present studies address some of the factors that, hopefully, can help further expand their use. Our studies demonstrate that CALA exhibits a unique activity towards various sterically hindered alcohols even including tertiary alcohols. These results indicate that CALA may open up for the use of lipase catalysis in reactions that are normally not considered compatible with the use of enzymes due to steric hindrance. With respect to CALB, a presently much more established catalyst within organic synthesis, we have shown that a new, potentially highly cost-effective, immobilization technology based on silica-granulation can provide a product with an activity and stability comparable to the commercially available immobilized preparations of this enzyme. This new product could, potentially, help further expand the large-scale use of this catalyst into areas where the price of the presently available products is prohibitive.

Experimental Section

Analytical Methods. ^{1}H NMR spectra were recorded on a Varian Mercury VX 400 MHz NMR spectrometer equipped with a 4N-ASW probe. Spectra were recorded in $CDCl_3$ at 30 °C using the $CHCl_3$ signal as internal standard. GC analysis was performed on a Varian Star 3400 CX equipped with a FID detector, helium as carrier gas and a Stabilwax-DA capillary column (30 m, 0.025 mm ID, Resteck). A starting temperature of 60 °C was used for 1 min and them an increase in 50 °C/min until reaching the final temperature of 250 °C that was kept for 3 min. In the case of very short-chained substrates, a starting temperature of 30 °C, a gradient of 20 °C/min and a final temperature of 200 °C were used.

Materials. All unspecified reagents were from commercial sources and of analytical grade. A reference of *iso*-propyl myristate was made in-house using traditional chemical methods. Precipitated hydrophilic silica powder (Sipernat 22), cellulose (Arbocel BFC-200), dextrin (Glucidex DE21), and Accurel EP 100 were obtained from Degussa, Rettenmaier and Roquette, and Akzo, respectively. Purified CALA and CALB concentrate was from Novozymes A/S and had enzymatic activities of 37.1 and 36.6 kLU/mL, respectively

Immobilization of CALA. CALA was immobilized on Accurel EP 100, a porous polypropylene matrix, as previously described.[24]A lipase load of 52 kLU/g was obtained. The final, dried immobilized preparation had a water content of 1% (w/w).

Esterification Using CALA as Catalyst. Solutions of the alcohol and the acid (0.4 M) were prepared in *n*-heptane. In the case of competition experiments, six different alcohols (always including 1-hexanol) were dissolved in individual concentrations of 66.7 mM each to yield a total alcohol-concentration of 0.4 M. Then 3.0 mL of the acid solution and 3.0 mL of the carboxylic acid solution were mixed, and 20 μL of cyclohexylbenzene was added as the internal standard. The experiments were initiated by the addition of 100 mg of immobilized CALA, and the reaction mixtures were stirred (30 rpm) at room temperature. Every 5 min 40 μL was withdrawn and mixed with 500 μL of *n*-heptane, and the progress of the reaction was analyzed by capillary GC analysis.

Measurement of Competitive Factors. The competitive factor α has been defined by for lipase-catalyzed esterifications.[28] Thus, if two substrates R1OH and R2OH compete for the enzyme, the ratio of the reaction rates for each substrate ($v1$ and $v2$) is at a given point in time related to the concentration of the substrates by:

$$\frac{v1}{v2} = \alpha\frac{[\mathrm{R1OH}]}{[\mathrm{R2OH}]}$$

The competitive factors were determined using 1-hexanol as reference based on the initial rates observed essentially as previously described.[20]

Granulation of CALB. The granulation was performed using a mechanical granulator (Lödige Ploughshare Mixer FM50, Paderborn, Germany). The operation was batch-wise. Silica-powder (5.00 kg) and cellulose (1.27 kg) were fed into the container manually and blended. Dextrin (1.32 kg) was dissolved in the CALB liquid concentrate (4.40 kg) and pumped into the granulation chamber through an atomization nozzle, and the granulation was started by applying mechanical shear forces with ploughs inside the granulator. After 15 min granulation time additional water (3.00 kg) was atomized, and agglomeration started to occur. During the following 10 min, agglomeration was increased, and final granules were building. After the granulation had occurred, the process was terminated, and the granulates were dried in a fluidized bed system (Uni Glatt, product temperature <40 °C), after which the granulates were dry (water content 2.5%). The final product (6.76 kg, theoretical yield: 8.28 kg) was sieved in the fraction 300−1000 μm.

Physical Characterization of Granulates. Surface area measurements (BET isotherm) were determined using a

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Micromeritics Gemini nitrogen adsorption instrument. The sieve fraction 425−710 μm was used for surface area determination. Porosity measurement (bed porosity) was performed by slowly immersing a known volume of the silica granules (50.0 mL, sieve fraction 300−1000 μm) into a known volume of *n*-heptane, ensuring that air bubbles can freely diffuse from the porous core of the particles. The change in total volume (*n*-heptane and granules) was determined and divided by the volume of the silica granules. The bed porosity includes both the internal pore volume and the space volume between the granules packed in a fix bed.

Assay for Free Lipase Activity (LU). Tributyrin was hydrolyzed under standard conditions at 30 °C, pH 7.0, and the activity was determined from the alkali consumption using a pH-stat (Radiometer, Denmark). The activity was given as LU (lipase unit), where 1 LU corresponds to the amount of lipase, which liberates 1 mmol butyric acid min^{-1}; 1 kLU = 1000 LU.

Synthesis of *iso*-Propyl Myristate (IPM). Myristic acid (4.75 g, 20.8 mmol) was dissolved in 2-propanol (5.01 g, 83.3 mmol) in a shaking water bath at 40 °C (150 rpm). Molecular sieves (1 g, 4 Å) and 1.00 g immobilized CALB (silica granulated or Novozym 435) were added to the reaction mixture. Samples of the reaction mixture were withdrawn after 0, 15, 30, 60, 120, 180, and 240 min reaction time, respectively, diluted directly in $CDCl_3$ and analyzed by ^1H NMR. The conversion of the reaction could be determined directly from the synthesis mixture by comparing integrals of the carboxylic acid and its *iso*-propyl ester, respectively. No conversion was observed in blind test without enzyme.

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