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Active-site titration analysis of surface influences on immobilized *Candida antarctica* lipase B activity☆

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ABSTRACT

Matrix morphology and surface polarity effects were investigated for *Candida antarctica* lipase B immobilization. Measurements were made of the amount of lipase immobilized and the catalyst's tributyrin hydrolysis activity, along with a determination of the lipase's functional fraction by active-site titration. Soluble, purified lipase had an active fraction of 84%. Immobilization on the hydrophobic surface of macroporous poly(methylmethacrylate) resin resulted in the full retention the lipase active fraction, while immobilization on the hydrophobic surface of mesoporous, amorphous, octyl-modified silica allowed retention of just half of the lipase active fraction. The polar surface of unmodified mesoporous, amorphous silica bound the lipase in such a manner that all of the immobilized enzyme was active. Mesoporous, crystalline SBA-15 silica also bound lipase so that it all was active. The polar, non-porous surface of fumed silica retained only a small fraction (28%) of active lipase. Substantial differences were found among the various supports in their ability to preserve catalytic activity upon vacuum drying. These findings demonstrate that surface polarity alone is not the only determinant for immobilization, as hydrophobic poly(methylmethacrylate) and hydrophilic SBA-15 were equally competent as lipase supports. The ordered-channel mesostructure of SBA-15 may provide a critical balance of interactions with the enzyme to preserve its native conformation.

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1. Introduction

The highly versatile *Candida antarctica* lipase B (CALB) is broadly employed in nonaqueous synthesis. The enzyme is used most often in an immobilized form. Immobilization facilitates enzyme reuse and downstream recovery of products. Commercially available immobilized CALB, such as Novozym 435 (in which the enzyme is adsorbed to an acrylic resin), shows long-term stability at moderately high temperatures and a tolerance for polar and non-polar solvents. Yet, studies regarding CALB immobilization continue to be generated at a rapid rate. These studies seek to improve catalyst performance by providing higher activity or greater substrate regio- and enantio-selectivity [1,2]. Lower cost is sometimes an additional goal [3–5]. Performance measures can be as simple as the catalytic rate per unit mass or volume of the catalyst, which may suffice for practical application. However, to understand the details of a particular immobilization process and how the enzyme responds to a given support material, it is necessary to additionally determine the amount of enzyme immobilized and how much of the immobilized enzyme remains catalytically active. With this information in hand, valid comparisons can be made among various techniques and supports.

Organophosphorus esters such as methyl 4-methylumbelliferyl hexylphosphonate (4-MUHP) were developed as sensitive active-site titrants for lipases [6]. The long-chain phosphonate group inactivates the lipase, while the highly fluorescent 4-methylumbelliferyl leaving group provides a stoichiometric measure of active enzyme. The technique can be applied to both soluble and immobilized lipase. Therefore, active-site titration may provide critical information regarding the immobilized lipase active fraction, when coupled with knowledge of the total amount of lipase in the catalyst preparation. Knowing the amount of active enzyme in a catalyst can be useful for comparing kinetic parameters under differing operating conditions [7] or among engineered variants [8].

Support morphology and surface chemistry are expected to greatly influence CALB activity [9]. Supports can be macroporous, mesoporous (2–50 nm pore diameter), microporous, or non-porous. Surface chemistries may include reactive species for covalent attachment, or be passive for simple adsorption of the

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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enzyme. Adsorption may be driven by electrostatic, polar and non-polar interactions between the support and enzyme. CALB has been immobilized by all such means, but there is generally insufficient information available to perform creditable comparisons between various supports and immobilization techniques. In the present work, active-site titration was applied to CALB immobilized on a sampling of adsorbent-type supports to determine how porosity and surface polarity influence the fraction of active enzyme.

2. Experimental

2.1. Reagents and materials

Novozym 435 (acrylic resin immobilized CALB), CALB (EC 3.1.1.3) in solution (Lipozyme CALB L), and Lewatit VP OC 1600 poly(methylmethacrylate) resin were obtained from Novozymes North America. Mesoporous silica (MS-3030) was kindly donated by PQ Corporation (Conshohocken, PA). CALB in the form of a highly purified enzyme powder (stated purity 95%) was purchased from Polium Technologies (Hoffman Estates, IL, USA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Inhibitor synthesis

Methyl 4-methylumbelliferyl hexylphosphonate was prepared using a procedure modified from Qian et al. [8]. To anhydrous toluene (300 mL) held in a stirred Schlenk flask on ice (under N_2), diisopropylethylamine (49.3 mmol, 8.58 mL), methanol (1.77 mL, 43.8 mmol), tetrazole (0.97 mL, 0.438 mmol), and hexylphosphonic dichloride (49.3 mmol, 8.44 mL) were added sequentially. The solution was warmed to room temperature and allowed to react for 4 h. To this solution was added a mixture of 4-methylumbelliferone (22.8 mmol; 7.71 g) and diisopropylethylamine (49.3 mmol, 8.58 mL) in toluene (30 mL). The combined reactants were stirred at room temperature for 16 h under a N_2 atmosphere. Unreacted 4-MU was removed by filtration through a fine glass frit. Solvent volume was reduced by rotary evaporation at 70 °C prior to product purification. 4-MUHP was purified by flash chromatography (octadecyl silica column; 90% methanol mobile phase) and semi-preparative HPLC (Phenomenex Luna phenyl–hexyl column; 35% methanol to 90% methanol gradient, then 90% methanol mobile phase) to yield 0.522 g of a clear oil pure by HPLC and NMR. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.56 (1 H, d), 7.23 (1 H, d), 7.18 (1 H, s), 6.23 (1 H, s), 3.82 (3 H, d), 2.42 (3 H, s), 1.93 (2 H, m), 1.68 (2 H, m), 1.40 (2 H, m), 1.3 (4 H, s), 0.88 (3 H, s). ^{13}C NMR ($CDCl_3$): δ (ppm) 160.5, 154.5, 153.2, 151.9, 125.8, 117.0, 116.9, 114.1, 108.9, 53.0, 31.2, 30.0, 26.0, 24.9, 22.2, 18.7, 13.9. Electron impact ionization gas chromatography/mass spectroscopy: 338 m/z (calcd $C_{17}H_{23}O_5P$: 338).

2.2.2. Octyl-silica and SBA-15 synthesis

MS-3030 silica was derivatized with octyltriethoxysilane in toluene as described previously [10]. The carbon content of the octyl-silica derivative was 5.15% by elemental analysis.

SBA-15 was prepared according to published methods [11]. To 250 mL of a 2 N HCl solution containing 3 wt% of the block copolymer P123 templating agent (Sigma–Aldrich) of average molecular weight 5800 were added 65 mmol tetraethylorthosilica (Sigma–Aldrich). This solution stirred at 50 °C for 18 h, and then aged, static, at 100 °C for 24 h. The solid was collected by filtration, washed with water and ethanol to remove the template, and then vacuum dried at 90 °C. The product was then calcined in air at 650 °C for 2 h.

2.2.3. Lipase purification and immobilization

Lipozyme CALB L (3 mL of 18.3 mg mL^{-1} CALB) was dialyzed (Pierce Slide-A-Lyzer dialysis cassette, 7000 MWCO) against 1 L of buffer (5 mM potassium phosphate, pH 7.0) for 6 h at 4 °C and then subjected to a brief, low speed centrifugation to remove insoluble material. The dialyzed CALB was adsorbed to Lewatit. The enzyme was extracted from the resin with 50% acetonitrile at 4 °C and then dialyzed extensively against buffer.

The immobilization support material (5–20 mg) in glass vials was first solvated with 1 mL of ethanol [12]. The support material was contacted with ethanol for 10 min, and then subjected to low-speed centrifugation to separate the support material from the ethanol. The pelleted material was washed three times with buffer (5 mM potassium phosphate, pH 7.0) to remove residual ethanol. Aliquots of CALB diluted to a concentration of 1.0 mg mL^{-1} in buffer were contacted with support material (5–20 mg) for 60 min at 23 °C, except for the Lewatit support, which was treated with CALB at 4 °C for 16 h. Sample vials were subjected to low-speed centrifugation to separate out the support material and immobilized lipase (for the purposes of this work, the combination of immobilized enzyme and support are referred to collectively as catalyst). Catalysts were washed three times with 1.0-mL aliquots of buffer, and then washed with *tert*-butanol. Supernatants were tested for residual protein content (Section 2.2.4) and lipase activity (Section 2.2.5). Catalysts were dried in a vacuum oven at 23 °C for 16 h. Catalysts were pulverized in *tert*-butanol for 60 min with a stir bar to reduce the support's particle size for lipase activity analysis (Section 2.2.5).

2.2.4. Protein analysis

Solution-phase CALB concentration was determined by the bicinchoninic acid method [13], using purified CALB powder to prepare calibration standards. Samples and standards were incubated at 37 °C for 30 min, allowed to cool to room temperature, and then absorbances were measured at 562 nm. Support-immobilized CALB quantification was conducted by treatment of a known dry mass of catalyst (typically 10 mg) with 10% formic acid/45% acetonitrile/45% water (1 mL) [14], followed by a bicinchoninic acid determination of the solubilized protein.

2.2.5. Tributyrin hydrolysis activity

Lipase catalyzed hydrolysis of tributyrin was followed titrimetrically in a Metrohm 842 Titrando pH-stat, using a 0.1 M NaOH titrant, for a period of 300 s. Lipase desorption from the various supports is negligible under these conditions [15]. The thermostated (28 °C) reaction vessel contained 60 mL of 5 mM potassium phosphate, pH 7.0, and 5 mL of tributryrin. Catalyst (5–20 mg) was pre-wetted with a small quantity of *tert*-butanol before being added to the vessel. One lipase unit (LU) corresponds to a titrant consumption rate of 1.0 μmol NaOH min^{-1}. Hydrolytic activity is reported as: whole catalyst activity (LU mg^{-1} catalyst), apparent specific activity (LU mg^{-1} protein), and specific activity (LU mg^{-1} active CALB). The whole catalyst value is based on the weight of immobilization support plus the weight of immobilized enzyme. Apparent specific activity is reported on a catalyst protein content basis and specific activity is based on the amount of catalytically active enzyme determined by active site titration (Section 2.2.6).

2.2.6. Active-site titration

The determination of the fraction of CALB molecules that were catalytically active was conducted using 4-MUHP and a procedure adopted from Qian et al. [8]. For CALB in solution (30 μM, 1 mg^{-1} mL), the lipase was treated with 60 μM 4-MUHP for 60 min at 23 °C in buffer (5 mM potassium phosphate, pH 7.0). For immobilized CALB, catalyst (5–20 mg) was solvated with 1 mL of acetonitrile containing 1% water and 60 μM 4-MUHP and then reacted for one week at 23 °C. At the end of the reaction period,

Table 1
Catalytic activities of CALB immobilized on various supports.

Support/catalyst	CALB load[a] (% w/w)	CALB active fraction (%)	Tributyrin hydrolysis activity[b]		
			LU (mg catalyst)$^{-1}$	LU (mg total CALB)$^{-1}$	LU (mg active CALB)$^{-1}$
Novozym 435	10	34 ± 1	10 ± 0.1	100	290 ± 10
Lewatit[c]	3.4	82 ± 6	10 ± 0.1	290	360 ± 30
Octyl silica[c]	4.8	39 ± 2	4.1 ± 0.2	86	220 ± 20
Octyl silica[c]	9.1	20 ± 1	4.8 ± 0.3	53	260 ± 20
Octyl silica[d]	9.1	48 ± 4	10 ± 0.3	110	230 ± 20
Octyl silica[c]	16.7	42 ± 3	15 ± 0.5	90	220 ± 20
Octyl silica[d]	16.7	40 ± 3	14 ± 0.5	82	210 ± 20
MS-3030 silica[d]	3.4	100 ± 1	9.2 ± 0.1	270	270 ± 10
SBA-15 silica[c]	3.1	98 ± 2	6.4 ± 0.4	210	210 ± 20
SBA-15 silica[d]	3.1	98 ± 2	6.8 ± 0.5	220	220 ± 20
Fumed silica[c]	4.0	ND[e]	1.6 ± 0.3	40	ND
Fumed silica[d]	4.0	28 ± 3	10 ± 0.2	250	890 ± 90

[a] Enzyme amount per entire catalyst (support + CALB) weight.
[b] Specific activity values (LU per milligram active CALB) where rounded off to two significant digits; standard deviations (one significant digit) were based on the larger of the error in the measurement of the CALB active fraction or hydrolysis rate. Relative standard deviations for CALB loading were 5% or less.
[c] Catalyst was room temperature vacuum dried following solvent exchange.
[d] Catalyst was solvent exchanged.
[e] Not determined.

the solvent was sampled for fluorometric determination of 4-methylumbelliferone (4-MU) released by the single turnover of each active CALB molecule. Note that enzyme does not need to be removed from its support for this analysis, only the 4-MU released from the reaction is collected and analyzed. The catalyst phase was extracted with acetonitrile, which preliminary experiments showed was able to fully desorb 4-MU from the enzyme support. Acetonitrile extracts and reaction solvent were combined. Aliquots were diluted into 10% acetonitrile/90% 0.1 M ammonia buffer (pH 9.5). Sample excitation was at 365 nm and fluorescence emission was measured at 448 nm (2 nm slit widths). A 4-MU linear calibration curve was constructed (15–90 nm). The concentration of 4-MU released from the lipase treatments was determined by regression. Control 4-MUHP solutions lacking enzyme (but with supports) were used to correct for background hydrolysis. Analyses were performed on catalysts in triplicate, for which mean and standard deviation values are reported.

2.2.7. Characterization of silica immobilization support textural properties

Solid sorbents were analyzed using a Quantachrome Autosorb surface area analyzer. Samples were degassed at 130 °C prior to analysis and analyses were performed at −196 °C. Surface areas were calculated from the BET equation in the pressure range of $0.05 < P/P_o < 0.25$. Mean pore diameter and volume were determined using the Barrett–Joyner–Halenda (BJH) method [16] on the desorption branch of the isotherm, except for SBA-15 silica for which the adsorption branch was used.

3. Results

3.1. Active-site titration of CALB in solution

A commercial sample of CALB in solution (Lipozyme®) was purified by selective adsorption and desorption using an acrylic resin (Section 2.2.3). The resulting enzyme preparation was determined to be electrophoretically pure (see Electronic Supplementary Information) [10,17]. Treatment of the purified CALB with 4-MUHP completely eliminated all detectible hydrolytic activity within 1 h. Based on the protein concentration of the enzyme solution and the amount of 4-MU released by 4-MUHP treatment of the enzyme, the active fraction of CALB was 84 ± 2%.

Under the condition of aqueous tributyrin hydrolysis, the purified CALB had an apparent specific activity 500 ± 20 LU mg^{-1} protein, and therefore a specific activity of 600 ± 20 LU mg^{-1} active CALB. It is to this specific activity value that comparisons with the immobilized enzyme forms were made. The determined specific activity (600 LU mg^{-1} active CALB is equivalent to a k_{cat} value of 330 s^{-1}) was similar to that observed by Larsen et al. [18] for purified wild type CALB (440 ± 110 s^{-1}).

3.2. Novozym 435 and purified CALB immobilization on acrylic resin

The widely studied commercial form of immobilized CALB, Novozym 435 was examined for comparison purposes. The protein content of the particular lot used for this study was determined to be 10% (w/w). Active site titration of CALB on this support (the acrylic resin Lewatit) cannot be performed in aqueous media as the inhibitor is largely adsorbed to the resin and thus does not have access to the enzyme. Therefore, active-site titrations conventionally have employed non-aqueous solvent for the reaction [19]. Preliminary studies using dry acetonitrile as the reaction solvent for the Lewatit-immobilized catalyst indicated that not all of the enzyme had been inactivated after two weeks of treatment with 4-MUHP. However, complete enzyme inhibition within a week was achieved using acetonitrile containing 1% (v/v) water, as recently reported by others [20]. It was found that 34% of Novozym 435's immobilized protein was catalytically active, which also agrees with a prior finding [20]. Note that a considerable lot-to-lot variation in the commercial version of the immobilized enzyme is to be expected. Gross and coworkers [21] observed a higher percentage of active enzyme (50%) in the material they examined.

To better compare CALB immobilization with various other supports, we examined the simple adsorption of purified CALB to Lewatit, apart from any proprietary co-solutes (if any) or processes that may be used to prepare Novozym 435. The catalyst protein load was 3.3% (w/w), low in comparison to the commercial version. The bound enzyme had the same active fraction of CALB as the soluble enzyme (Table 1). Specific activities of either catalyst (Novozym 435 or pure CALB on Lewatit) were quite similar (Table 1). Despite the high retention of active enzyme during immobilization, the specific activity of CALB on Lewatit was just little more than half of that of the soluble enzyme. The loss of hydrolytic activity upon immobilization may be ascribed to the imposition of substrate internal diffusion limitations or to a slight alteration of the enzyme's conformation so as to lower its catalytic proficiency, but it was not due to a partial loss of active enzyme in this case.

Table 2
Textural properties of silica-based immobilization supports.

Support	Surface area[a] ($m^2\ g^{-1}$)	Pore volume[b] ($cm^3\ g^{-1}$)	Pore diameter (nm)
MS-3030	280	2.97	33.6[b]
Octyl silica	294	2.98	31.1[b]
SBA-15	900	1.25	9.0[c]
Fumed silica	219	0.81	14.5[b]

[a] Multipoint BET method.
[b] BJH cumulative desorption method.
[c] BJH cumulative adsorption method.

3.3. CALB immobilization on silica

Silicas present an adsorption environment that is substantially different from that of organic resins such as Lewatit. Table 2 presents a summary of the physical attributes of the various silicas used to immobilize CALB in the present study. MS-3030 silica and its octyl derivative had similarly large surface areas and nearly the same pore diameters. A CALB molecule (approx. 5 nm in diameter) readily fits into these pores. SBA-15 had a surface area much higher than the other immobilization supports, including Lewatit ($100–150\ m^2\ g^{-1}$ [22]), as well as the smallest pore diameter of all the silicas. The determined 9-nm pore diameter of this SBA-15 preparation was identical to that reported by Forde et al. [23], although the surface area and pore volume are somewhat larger for the material used herein. Fumed silica had the smallest surface area and pore volume of all the examined materials. It is likely that CALB adsorption would be mostly limited to exterior surfaces of SBA-15 and fumed silica.

3.3.1. Octyl silica

Octyl silica demonstrated an impressively high capacity for adsorbing CALB, as reported previously [10,12]. Pre-solvating the octyl silica with ethanol (Section 2.2.3) obviated the need to have ethanol present during CALB immobilization. Even with a 20% CALB load offering (e.g., 4 mg CALB present to 20 mg of octyl silica), all of the enzyme was adsorbed (16.7% (w/w) of CALB in the catalyst), as there was no detectable protein or hydrolytic activity remaining in the aqueous phase after the adsorption step. This was fortuitous because the protocol used to measure bound protein (formic acid/acetonitrile/water extraction) did not quantitatively remove CALB from this support, unlike that found with the other silicas and acrylic resin. CALB must be very tightly bound to this adsorbent.

The catalytic activity of CALB adsorbed onto octyl silica was examined over a range of enzyme loading levels (Table 1). As in the case of CALB adsorbed to Lewatit, active-site titration in acetonitrile containing 1% (w/w) water was necessary and sufficient to determine the fraction of active CALB molecules adsorbed to this support. When the catalyst was vacuum dried, the fraction of active CALB was just half, at best, of that of the soluble enzyme. Surprisingly, the active fraction (20%) of the catalyst with a 9.09% (w/w) enzyme content was significantly lower than that those with either higher or lower enzyme loads. This curious observation, not previously noted [10,12], was examined further.

As an alternative method to vacuum drying of the catalyst, a solvent-exchange procedure was developed. Following enzyme adsorption, the catalyst was rinsed with *tert*-butanol, followed by an acetonitrile rinse. Thus, the catalyst was not dried before being submitted to hydrolytic activity and active-site titration analyses. When the solvent-exchange procedure was applied to the 9.09% (w/w) enzyme-loaded octyl silica catalyst the active fraction of CALB was twice as high as the vacuum dried sample. Conversely, the same protocol applied to 16.7% (w/w) loaded octyl silica did not alter its active fraction.

3.3.2. MS-3030 silica

The MS-3030 silica used to prepare octyl silica also had a significant binding capacity for CALB. An enzyme load of 3.4% (w/w) was readily achieved, which compares closely with that obtained using Lewatit as the immobilization support. It was found that active-site titration of CALB adsorbed onto MS-3030 silica could be readily conducted on the catalyst under aqueous conditions, just as that performed on the soluble enzyme (1 h, room temperature, 5 mM potassium phosphate buffer, pH 7.0), with an additional acetonitrile extraction of the catalyst step added following the reaction with 4-MUHP to ensure complete recovery of the 4-MU. Active-site titration of the MS-3030 silica-immobilized CALB indicated that 100% of the enzyme was catalytically active, even though the applied CALB was only 84% active. This result suggests that the silica's surface may induce a beneficial conformational change in the inactive CALB fraction upon adsorption.

The specific activity of MS-3030 silica-immobilized CALB was comparable to that found for the octyl silica and Lewatit catalysts (Table 1). As it is unlikely that macroporous Lewatit (approx. 100 nm pore diameter [24]) and mesoporous MS-3030 silica (or octyl silica) present the same internal mass transport limitation. Therefore, the finding of similar specific activities, all of which are much less than that of the soluble enzyme, indicates that the impact of immobilization is on CALB's conformational flexibility during the catalytic cycle. Despite this highly efficacious immobilization, the MS-3030 silica-based catalyst retained none of its activity upon drying.

3.3.3. SBA-15 silica

Although the surface area of SBA-15 far exceeded that of the other immobilization supports (Table 2), its CALB loading capacity (3.1% (w/w)) was quite similar to that of MS-3030 silica and Lewatit. The fraction of active immobilized enzyme was also very high (Table 1) when the active-site titration was performed in aqueous media without prior vacuum drying of the catalyst. The activity of catalyst was unchanged by solvent exchange (*tert*-butanol) or vacuum drying. Thus the SBA-15 silica surface, unlike that of MS-3030 silica, allowed the enzyme to retain a reactive conformation after drying the catalyst.

SBA-15's pores are only slightly larger than the enzyme, on average. Measurement of SBA-15 textural properties after CALB adsorption indicated there was a 23% pore volume decrease with the CALB present (see ESI Fig. S2). One interpretation of this finding is that a portion of the support's pores was occluded by CALB.

3.3.4. Fumed silica

Despite the small pore diameter (14 nm) of the fumed silica, it too was able to adsorb a considerable amount of CALB, 4.0% (w/w). This finding is consistent with the concept developed by Cruz et al. [25] that CALB adsorbes to the exposed surfaces of ~10 nm nanoparticles that comprise the fumed silica agglomerate. The fraction of active CALB in the catalyst (28%) was the lowest among the support materials examined herein, which is consistent with the high extent of enzyme unfolding observed by fluorescence changes when placed in contact with fumed silica [26]. The hydrolytic activity ($LU\ mg^{-1}$ total CALB) of the fumed silica catalyst was similar to that of MS-3030 and SBA-15 silicas (Table 1), as long as the catalyst was not vacuum dried. The activity of the vacuum dried catalyst was quite impaired ($40\ LU\ mg^{-1}$ total CALB compared to $250\ LU\ mg^{-1}$ total CALB for the solvent exchanged version). The specific activity of the solvent exchanged fumed silica catalyst was several-fold higher than that of the other silica-immobilized CALBs (Table 1), and in fact 50% greater than the activity of CALB free in solution. Fumed silica itself displayed no hydrolytic activity. The immobilized CALB activity was completely inhibited by 4-MUHP treatment in buffer, which indicated that the fraction of active

CALB in the catalyst was not underestimated. It is unclear whether the increased specific activity is due to surface-induced changes to the enzyme or greater substrate availability (tributyrin is poorly soluble under the conditions of the hydrolysis assay). CALB has a small active-site lid region, so it would not be expected to demonstrate significant interfacial activation [27]. Prior studies showing modest catalytic activity enhancement upon immobilization have been with hydrophobic surfaces [23,28,29]. A recent study showed that CALB adsorbed to fumed silica had greater transesterification activity than a comparable amount of Novozym 435 CALB [30]. The apparent hyperactivation of CALB adsorbed to the polar surface of fumed silica reported in the present work thus represents a creditable observation.

4. Discussion

Active-site titration with 4-MUHP illustrated the great range of potential surface impacts on CALB activity. Depending on the surface, immobilization can increase (MS-3030 and SBA-15 silicas), fully retain (Lewatit), or substantially decrease (octyl and fumed silicas) the fraction of active enzyme (Table 1). A support surface can largely protect the absorbed enzyme from denaturation during a drying process (Lewatit and SBA-15), or not do so (MS-3030 and fumed silicas). The balance between protein–surface and protein–protein interactions is a second order influence, such as in the case of octyl silica in which the drying process impacted only a specific enzyme adsorption level (9.1% (w/w)).

The impact of immobilization on the enzyme's active fraction is distinctly different from that on specific activity. CALB immobilized on macroporous Lewatit can fully retain the enzyme's active fraction, although not its specific activity (approx. half of the soluble enzyme's specific activity). Conversely, the effectively non-porous fumed silica had a specific activity markedly higher than that of the soluble enzyme (Table 1), but demonstrated poor retention of enzyme active fraction. Such differences would not be apparent based on measures of activity per unit of catalyst or per unit of total enzyme. For instance, by these measures the Lewatit and fumed silica preparations would appear to be identical (Table 1). Active-site titration thus provides new insight into the influence of surface chemistry on CALB immobilization. Such information would be useful for interpreting molecular-level spectroscopic measurements (e.g., NMR, circular dichroism spectroscopy) on an entire assemblage of immobilized enzyme with an otherwise unknown active fraction.

Chen et al. [31] performed active-site titration of CALB adsorbed to mesoporous (25-nm pore diameter) poly(methylmethacrylate) and found 30–45% of the immobilized CALB was reactive (although there no indication given of the active fraction of the CALB used for immobilization). Even lower active fractions were obtained (14–25%) when covalent attachment to this support was attempted [21]. Therefore physical adsorption of CALB on macroporous poly(methylmethacrylate) resin (Lewatit) appears to be more successful (full retention of active lipase fraction, Table 1).

Serra et al. [32] described the textural properties of MS-3030 silica as amorphous mesoporous and SBA-15 as ordered mesoporous. They reported CALB loadings of 4.5% and 4.4%, respectively, values that are slightly higher than those found in the present work (Table 1). Both materials impart a very high degree of native structure on CALB and the catalysts display similar specific activities. However, the materials have distinctly different impacts on the enzyme when subjected to drying (the SBA-15-based catalyst retains activity while MS-3030 silica catalyst does not). The p*I* of silica-based mesoporous materials is approximately 2 [33], while the isoelectric region of CALB is broadly from pH 5 to 8 [34]. Consequently, at pH 7 (the immobilization pH), SBA-15 and MS-3030 silica carry a negative charge and portions of CALB's surface is positively charged [34], so an electrostatic interaction between enzyme and support is anticipated to be the driving force for immobilization with either material. Forde and coworkers [23] found that CALB adsorption to SBA-15 occluded the support's pores. They concluded that close packing within the pores gave rise to enhanced enzyme thermostability. The ordered pores of SBA-15 must present an architecture that prevents CALB denaturation upon drying, while the amorphous features of MS-3030 silica do not. The size of CALB (35 kDa, including glycosyl groups [14]) approaches the upper limit of biomolecules (43 kDa) that can penetrate SBA-15 pores [33]. Therefore it is reasonable to envision a pore-entrapped CALB molecule largely surrounded by a silica surface that does not distort the shape of the enzyme.

It is interesting to consider what feature of SBA-15 is responsible for CALB adsorption. With a CALB loading of 31 mg g^{-1} (Table 1) and 0.5 m^2 mg^{-1} projected CALB surface area, the adsorbed enzyme covers but 1.7% of the support surface (see Supporting Electronic Information for calculations). That such a small amount of coverage can result in a 23% reduction in pore volume (Section 3.3.3) raises the concept of one or two CALB molecules occupying a pore opening, leaving the pore interior enzyme free.

Fumed silica did not support a high native conformation of CALB upon immobilization, nor after subsequent drying. Fumed silica is an amorphous material comprising fused, non-porous nanoparticles. Cruz and coworkers used a somewhat different protocol from the one employed herein for CALB immobilization on fused silica [25]. In Cruz et al., after enzyme adsorption the catalyst was lyophilized, rather than solvent exchanged and vacuum dried at room temperature [25]. They observed that catalytic activity was greatly sensitive to the enzyme-to-silica ratio, with 1:9 (w/w) (i.e., 10% CALB, 90% fumed silica) being optimal. The 4% loading achieved in the present work was therefore below the optimal level, which may account for the low active CALB fraction (28%) observed. They postulated that optimum surface coverage led to a predominance of protein–protein interactions over multi-point protein–silica attachments. Their CALB-fumed silica preparation did not display substrate diffusion limits, which is consistent with the exceptionally high specific activity observed herein for the catalyst. However, it was shown by atomic force microscopy that CALB adsorbing to a planar hydrophilic surface results in multi-layer formation [28], so the active protein layer may in fact not be in direct contact with a hydrophilic silica surface, but rather it is adsorbed to an underlying denatured protein layer.

Gao et al. [35] examined CALB adsorption to methyl-modified silica aerogel (38-nm pore diameter). This material has a hydrophobic surface. It had a high loading capacity (210 mg of CALB per gram of support, therefore 17% (w/w) of the catalyst). However, no indication was given of how the reported activity (olive oil hydrolysis) of the immobilized enzyme compared to that of the soluble protein. These authors reported that a 90% (v/v) *n*-butanol rinse of the catalyst increased its activity 2.6-fold. Although the octyl silica surface should be similar to methyl-modified silica, no such large solvent activation of the immobilized enzyme activity was observed in the present work. This may be reflect small but important differences in the treatment protocols, as they found solvent treatment (100% *n*-butanol) and subsequent air drying led to a substantial loss of activity (approx. 75% less). Vacuum drying the octyl silica catalyst did not lower its activity at most loading levels (Table 1). The present work confirms that details of solvent exchange and drying, as well as enzyme packing density, are important aspects of CALB immobilization on hydrophobic silicas.

From a practical viewpoint, octyl silica was the best performing support (based on hydrolytic activity per unit mass of catalyst; Table 1) due to its very high adsorption capacity for CALB. Reactor productivity is important for large-scale biocatalysis. Space–time

yield or volumetric productivity is critically influenced by immobilized enzyme concentration. The octyl silica-based catalyst would excel by this metric. Furthermore, the enzyme forms an intense interaction with this support, which prevents enzyme leaching under extended use [10,12].

5. Conclusions

Active-site titration revealed that CALB immobilization is sensitive to support surface chemistry, pore size and structure, and enzyme packing density. Hydrophobic, mesoporous octyl silica excels as a carrier because of its unmatched adsorption capacity for CALB, but an unidentified attribute leading to some denaturation, perhaps overly strong enzyme adsorption, detracts from its value as a support. Hydrophilic, mesoporous silica was highly conducive for preserving the native structure of CALB, and apparently helped refold the enzyme as well. This is unexpected given prior findings that CALB bound to a flat, hydrophilic surface denatures to a great extent [28]. Non-porous fumed silica suffers similarly from its inability to preserve the enzyme's natural state. These findings imply that multiple interactions between silica walls and protein in a pore setting can stabilize protein structure. The ordered-channel mesostructure of SBA-15 was particularly efficacious in preserving activity even under harsh processing conditions. SBA-15 or octyl silica may be suitable alternatives to acrylic resin as supports for CALB in non-aqueous applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.12.011.

References

[1] J.M. Palomo, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisan, Tetrahedron: Asymmetry 13 (2002) 2375–2381.
[2] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[3] D.S. Rodrigues, A.A. Mendes, W.S. Adriano, L.R.B. Gonçalves, R.L.C. Giordano, J. Mol. Catal. B: Enzym. 51 (2008) 100–109.
[4] A. Brígida, Á. Pinheiro, A. Ferreira, L. Gonçalves, Appl. Biochem. Biotechnol. 146 (2008) 173–187.
[5] M. Würtz Christensen, L. Andersen, T.L. Husum, O. Kirk, Eur. J. Lipid Sci. Technol. 105 (2003) 318–321.
[6] R. Fujii, Y. Utsunomiya, J. Hiratake, A. Sogabe, K. Sakata, Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids 1631 (2003) 197–205.
[7] L.F. García-Alles, V. Gotor, Biotechnol. Bioeng. 59 (1998) 684–694.
[8] Z. Qian, C.J. Fields, S. Lutz, ChemBioChem 8 (2007) 1989–1996.
[9] U. Hanefeld, L. Gardossi, E. Magner, Chem. Soc. Rev. 38 (2009) 453–468.
[10] R.M. Blanco, P. Terreros, M. Fernández-Pérez, C. Otero, G. Díaz-González, J. Mol. Catal. B: Enzym. 30 (2004) 83–93.
[11] D. Zhao, J. Feng, Q. Huo, N. Melosh, G.H. Fredrickson, B.F. Chmelka, G.D. Stucky, Science 279 (1998) 548–552.
[12] R.M. Blanco, P. Terreros, N. Muñoz, E. Serra, J. Mol. Catal. B: Enzym. 47 (2007) 13–20.
[13] C.M. Stoscheck, Methods Enzymol. 182 (1990) 50–68.
[14] I. Petry, A. Ganesan, A. Pitt, B.D. Moore, P.J. Hailing, Biotechnol. Bioeng. 95 (2006) 984–991.
[15] E. Serra, E. Díez, I. Díaz, R.M. Blanco, Micropor. Mesopor. Mater. 132 (2010) 487–493.
[16] E.P. Barrett, L.G. Joyner, P.P. Halenda, J. Am. Chem. Soc. 73 (1951) 373–380.
[17] J.A. Laszlo, K.O. Evans, J. Mol. Catal. B: Enzym. 58 (2009) 169–174.
[18] M.W. Larsen, U.T. Bornscheuer, K. Hult, Protein Expr. Purif. 62 (2008) 90–97.
[19] D. Rotticci, T. Norin, K. Hult, M. Martinelle, Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids 1483 (2000) 132–140.
[20] C. Hedfors, K. Hult, M. Martinelle, J. Mol. Catal. B: Enzym. 66 (2010) 120–123.
[21] B. Chen, J. Hu, E.M. Miller, W. Xie, M. Cai, R.A. Gross, Biomacromolecules 9 (2008) 463–471.
[22] T. Nakaoki, Y. Mei, L.M. Miller, A. Kumar, B. Kalra, M.E. Miller, O. Kirk, M. Christensen, R.A. Gross, Ind. Biotechnol. 1 (2005) 126–134.
[23] J. Forde, A. Vakurov, T.D. Gibson, P. Millner, M. Whelehan, I.W. Marison, C. Ó'Fágáin, J. Mol. Catal. B: Enzym. 66 (2010) 203–209.
[24] Y. Mei, L. Miller, W. Gao, R.A. Gross, Biomacromolecules 4 (2002) 70–74.
[25] J.C. Cruz, P.H. Pfromm, M.E. Rezac, Process Biochem. 44 (2009) 62–69.
[26] J.C. Cruz, P.H. Pfromm, J.M. Tomich, M.E. Rezac, Colloids Surf. B: Biointerfaces 79 (2010) 97–104.
[27] M. Martinelle, M. Holmquist, K. Hult, Biochim. Biophys. Acta – Lipids Lipid Metab. 1258 (1995) 272–276.
[28] J.A. Laszlo, K.O. Evans, J. Mol. Catal. B: Enzym. 48 (2007) 84–89.
[29] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisán, J. Mol. Catal. B: Enzym. 19–20 (2002) 279–286.
[30] M. Kramer, J.C. Cruz, P.H. Pfromm, M.E. Rezac, P. Czermak, J. Biotechnol. 150 (2010) 80–86.
[31] B. Chen, E.M. Miller, L. Miller, J.J. Maikner, R.A. Gross, Langmuir 23 (2006) 1381–1387.
[32] E. Serra, Á. Mayoral, Y. Sakamoto, R.M. Blanco, I. Díaz, Micropor. Mesopor. Mater. 114 (2008) 201–213.
[33] H.H.P. Yiu, P.A. Wright, J. Mater. Chem. 15 (2005) 3690–3700.
[34] P. Trodler, J. Nieveler, M. Rusnak, R.D. Schmid, J. Pleiss, J. Chromatogr. A 1179 (2008) 161–167.
[35] S. Gao, W. Wang, Y. Wang, G. Luo, Y. Dai, Bioresour. Technol. 101 (2010) 7231–7238.