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Influence of self-assembled monolayer surface chemistry on *Candida antarctica* lipase B adsorption and specific activity☆

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Abstract

Immobilization of *Candida antarctica* B lipase was examined on gold surfaces modified with either methyl- or hydroxyl-terminated self-assembled alkylthiol monolayers (SAMs), representing hydrophobic and hydrophilic surfaces, respectively. Lipase adsorption was monitored gravimetrically using a quartz crystal microbalance. Lipase activity was determined colorimetrically by following *p*-nitrophenol propionate hydrolysis. Adsorbed lipase topography was examined by atomic force microscopy. The extent of lipase adsorption was nearly identical on either surface (approximately 240 ng cm^{-2}), but its specific activity was sixfold higher on the methyl-terminated SAM, showing no activity loss upon immobilization. A uniform, 5.5 nm high, highly packed monolayer of CALB formed on the methyl-terminated SAM, while the adsorbed protein was disordered on the hydroxyl-terminated SAM. Hydrophobic surfaces thus may specifically orient the lipase in a highly active state.
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Keywords: Protein adsorption; Lipase; Immobilization

1. Introduction

Nonaqueous biocatalysis is finding commercial utility in the production of fine and specialty chemicals [1–3]. Lipases are among the most broadly deployed biocatalysts because of their ability to produce chiral chemicals with high enantiomeric purity [4]. Lipases catalyze hydrolysis, alcoholysis, esterification and transesterification of carboxylic acids or esters. The B lipase from *Candida antarctica* (CALB) has been a synthesis workhorse, as well as the subject of numerous fundamental studies regarding nonaqueous enzymology [5–7]. CALB typically is used in an immobilized form, such as the commercial product Novozym 435.

Enzyme immobilization offers many potential benefits. Immobilization can improve enzyme operational performance and stability, as well as provide for ready separation of biocatalyst from the reaction medium [8,9]. CALB has been immobilized on various support materials such as porous resins and silicas [5,10–15]. Polypropylene and acrylic resins, regarded as hydrophobic supports, are particularly efficacious. These meso- and macroporous polymeric materials accommodate high CALB loadings (up to 20% w/w protein) and good retention of enzyme activity. It is not understood how the support matrix influences CALB activity. The support matrix can impose activity limitations by altering the lipase's native conformation or by diminishing substrate diffusivity. Distinguishing between these influences can be difficult. Infrared detection of CALB on various supports indicates a very heterogenous distribution within the polymer matrix [12,16]. Potentially, there are substantial amounts of lipase that do not interact directly with the polymer surface. Thus, conventional immobilization materials do not provide a sufficiently uniform enzyme–support interface for study of support surface influences on CALB activity and topography.

The current study examines the impact of support surface properties (hydrophobicity) on CALB activity, in an arrangement that does not impose substrate internal transport limitations, through the use of flat, self-assembled alkylthiol-modified gold surfaces [17]. A correlation between surface

☆ 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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hydrophobicity and enzyme topographical arrangement is evaluated.

2. Experimental

2.1. Reagents and materials

CALB in solution (commercial name Lipozyme CALB-L) was obtained from Novozymes North America. CALB in the form of a highly purified enzyme powder (stated purity 95%) was purchased from Polium Technologies (Hoffman Estates, Illinois, USA), as was *p*-nitrophenylpropionate (pNPP). The powder form of CALB was used solely in the preparation of protein calibration standards for determining the CALB concentration in the liquid sample. 1-Undecanethiol (MU), 11-mercapto-1-undecanol (MUOH), and ethanol were from Sigma–Aldrich. Water was obtained from a Barnstead NANOpure Diamond UV ultrapure water purification system (resistivity 18.2 MΩ cm).

2.2. Self-assembled monolayer (SAM) preparation

The Au surfaces of QCM sensor crystals (Q-Sense) and Au-coated glass slides (Platypus Technologies) were cleaned sequentially by UV/O_3 treatment (15 min), $H_2O/NH_4OH/H_2O_2$ (5:1:1 v/v/v) at 70 °C (15 min), another UV/O_3 treatment (15 min), and finished with rinses of water and then ethanol. Au-covered mica (SPI Supplies) was not subjected to the $H_2O/NH_4OH/H_2O_2$ cleaning step but instead was given water and methanol rinses in between UV/O_3 treatments. Cleaned substrates were immersed for at least 24 h in ethanolic solutions of 10 mM MU or MUOH, respectively forming methyl- or hydroxyl-terminated SAMs. SAM surfaces were rinsed with ethanol and dried under a stream of N_2. Static water contact angles on SAM-modified Au-coated glass slides were measured immediately using a FTA200 optical system (First Ten Angstroms Inc., Portsmouth, VA).

2.3. Protein and hydrolytic activity assays

CALB concentration in the solution received from Novozymes was determined by the bicinchoninic acid method [18,19], 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 their absorbances were measured at 562 nm.

CALB specific activity was assessed by following the catalytic generation of the *p*-nitrophenolate anion (15,000 M^{-1} cm^{-1} at 410 nm) from pNPP hydrolysis [20]. One unit (U) of lipase activity produces 1 mol of product per min. Reactions were conducted in 10 mM $KHPO_4$, pH 7.0, buffer containing 1.0 mM pNPP at 25 °C. Buffer-solubilized CALB activity was determined in 1-cm-pathlength cuvettes using a Shimadzu 1240 UV–vis spectrophotometer with the enzyme at a concentration of 4.5 μg/mL. Color development was linear with time for several minutes. pNPP hydrolysis in the absence of CALB was negligible. To determine the activity of surface-immobilized CALB, SAM-modified Au-coated glass slides (25 mm × 75 mm) were incubated for 3 h at room temperature in buffer containing 450 μg/mL (13.5 μM) CALB. Slides were transferred to buffer for 2 min to remove loosely adhered protein, then briefly rinsed with a stream of water. Excess water was wicked from the surface. To create reaction wells on the slides, flat-sided glass O-rings (2.25 cm i.d. and 0.5 cm high) were attached to the slides (two O-rings per slide) with vacuum grease. Each O-ring enclosed 4.0 cm^2 of slide surface. The constructed wells were filled with 1.0 mL of buffer containing 1.0 mM pNPP and the slides were placed in a forced-air orbital shaker operating at 90 rpm and 25 °C. Slides were covered with a Petri dish to minimize fluid evaporation from the wells. At timed intervals, well reaction medium (1.0 mL) was transferred to cuvettes for photometric analysis of the reaction product concentration. Because the reaction with immobilized CALB evolved more slowly than with CALB in solution, a slight amount of uncatalyzed product formation was observed to occur. Therefore, a control solution of pNPP was used to subtract background, uncatalyzed nitrophenolate anion generation from the CALB-catalyzed reaction. The uncatalyzed rate was approximately 7% of that of the slowest catalyzed reaction, i.e., with CALB immobilized on a hydroxyl-terminated SAM (see Section 3.3). Four time points per reaction were taken (i.e., using two slides), typically ranging from 2 to 20 min contact time of the reaction buffer with the slide surface. No pNPP hydrolysis (other than at the uncatalyzed hydrolysis rate) was observed with SAM-modified surfaces lacking adsorbed CALB. Analysis of CALB activity on each SAM surface (methyl- and hydroxyl-terminated) was performed four to six times.

2.4. Quartz crystal microbalance (QCM) measurements

QCM measurements of CALB adsorption to SAM-modified Au surfaces on AT-cut quartz crystals were performed with a Q-Sense D300 system (Q-Sense Inc., Glen Burnie, MD). The crystal and solution chamber temperature was maintained at 25.0 °C. The QCM technique, described in detail elsewhere [21,22], provides information about the amount of adsorbed mass through changes in vibrational frequency (*f*). For rigid films, such as that obtained with the adsorbate dried onto the crystal surface (see below), the Sauerbrey equation [21] can be employed to determine adsorbed mass (Δm):

$$\Delta m = -\left(\frac{C}{N}\right)\Delta f \quad (1)$$

where *C* is the mass sensitivity constant, 17.7 ng cm^{-2} Hz^{-1} the primary harmonic (5 MHz) and *N* is the overtone number. The 15 MHz ($N = 3$) overtone was used for quantifying protein dry mass (ng cm^{-2}).

SAM-modified crystals were assembled into the QCM unit, and then the cell was flushed 20 min with N_2. The system was allowed to equilibrate overnight at temperature, which was necessary to eliminate drift in *f*. The cell was flushed again with N_2 (20 min), and the absolute *f* of the third overtone was recorded. Buffer (10 mM $KHPO_4$, pH 7.0, 0.2 μm filtered and degassed) was passed into the cell to establish baseline values of *f* in liquid.

This was followed by a solution of CALB (450 μg/mL) in buffer, which allowed CALB to adsorb onto the crystal surface under non-flow conditions, and Δf was continuously monitored in the conventional manner. The cell was then successively flushed with water, to remove buffer salts (no changes in f and D apparent), N_2 (20 min), ethanol (5 mL) and again with N_2 (20 min) to provide a dry protein film on the crystal surface and the absolute f (under N_2) was recorded. The decrease in f (Δf) was translated to Δm using Eq. (1). Preliminary experiments showed that, absent CALB adsorption, this treatment regimen had minimal impact on measurements in air ($\Delta f < 3$ Hz).

2.5. Atomic force microscopy (AFM)

CALB adsorbed to SAM-modified Au on mica was imaged in air using tapping mode AFM with a Nanoscope IV (Veeco Metrology) instrument equipped with a diamond-like carbon spike (DLCS) probe (1–3 nm tip radius).

2.6. Molecular modeling

The CALB crystal structure [23] 1TCA was downloaded from the Protein Data Bank. RasTop version 2.0.3 was used for rendering and intramolecular distance measurement.

3. Results and discussion

3.1. Surface characterization

The influence of surface hydrophobicity on CALB adsorption and activity was examined. Self-assembly of alkane thiols on Au surface provides a method of forming surfaces with uniform properties. Water contact angle measurements indicated that hydroxyl- and methyl-terminated SAM surfaces were hydrophilic (20°) and hydrophobic (75°), respectively. This large difference in surface hydrophobicity was considered adequate to compare its effect on CALB adsorption and activity.

3.2. QCM analysis of CALB adsorption

CALB adsorption to a methyl-terminated SAM as observed by the QCM technique occurred in two kinetic regimes (Fig. 1). Three-fourths of the final frequency shift occurred within the mixing time of the cell (<2 min), which was followed by a very slow adsorption phase extending almost to 180 min, at which point no further adsorbed mass increase was observed. Rinsing the cell with buffer removed loosely adhered protein (small increases in f). CALB adsorption to a hydroxyl-terminated SAM displayed a similar QCM response (Fig. 1). With either surface, CALB showed no indication of desorbing in buffer after the cell rinse, indicating that stable protein films were obtainable with either hydrophobic or hydrophilic surfaces. The Δf values after the buffer rinse were not significantly different for the two surfaces (110 ± 10 and 137 ± 25 Hz, respectively, for the methyl- and hydroxyl-terminated SAM surfaces), indicating that approximately similar amounts of mass adsorbed in each case.

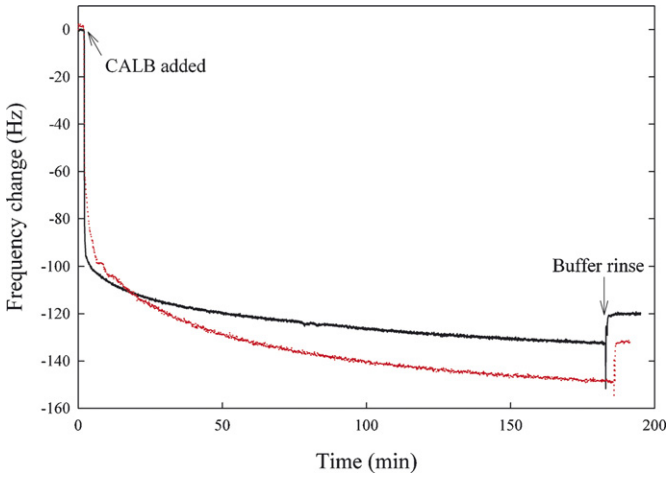

Fig. 1. Representative QCM CALB adsorption traces with methyl- and hydroxyl-terminated SAM surfaces (solid black and dotted red lines, respectively). Frequency change (Δf) was monitored at the crystal's third overtone (approximately 15 MHz).

For proteins adsorbed from buffer onto a surface, the adsorbed mass observed reflects contributions from protein and acoustically coupled water when measured by the QCM technique. To provide a precise determination of adsorbed CALB mass alone, the cell and crystal surface were dried and the resulting Δf value was determined in atmosphere. Applying the Sauerbrey Eq. (1) to CALB adsorbed to a methyl-terminated SAM surface following a 180 min equilibrium period indicated an adsorbed CALB mass of 242 ± 38 ng cm^{-2}. The hydroxyl-terminated SAM had a similar amount of CALB dry mass adsorbed to it (244 ± 115 ng cm^{-2}). Therefore, the CALB dry mass values correlated with the observed hydrated mass Δf values (Fig. 1). Wannerberger and Arnebrant [24] estimated closed packed monolayer coverage would be 274 ng cm^{-2} for the lipase with a 4.0 nm × 5.0 nm surface orientation. The observed extent of CALB binding on either surface thus was consistent with near monolayer coverage.

Commercially sourced CALB shows a single band by SDS-PAGE [16,25,26], so the observed QCM response cannot be attributed to other proteins. However, unidentified excipients in the commercial preparation, although 100-fold diluted for these experiments, could not be precluded as a contributing factor in the protein's adsorption behavior.

3.3. Immobilized CALB activity

CALB displayed dramatically different hydrolytic activities when immobilized on methyl- and hydroxyl-terminated SAM surfaces (Fig. 2). The QCM results demonstrated that once adsorbed to a SAM surface and buffer rinsed, no further CALB desorption in buffer occurs. Thus, hydrolytic activity associated with CALB detaching from the surface was not a concern. The hydrolysis rate with CALB on the methyl-terminated SAM surface was sixfold higher than with it on the hydroxyl-terminated SAM surface. Assuming 240 ng cm^{-2} of CALB on either surface, the specific activities were 5.4 and

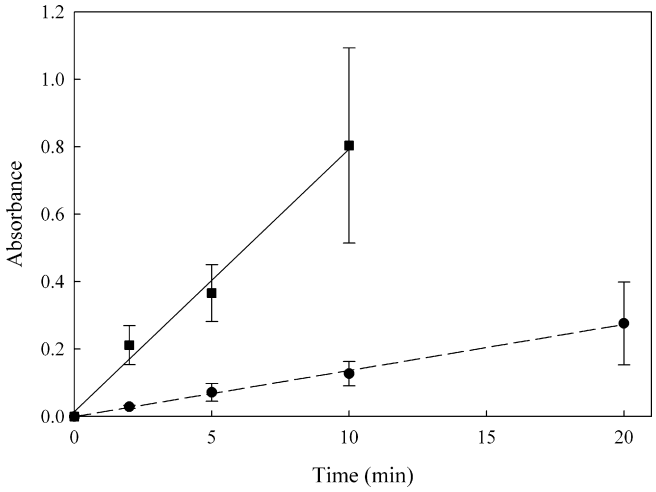

Fig. 2. pNPP hydrolysis by CALB immobilized on methyl- and hydroxyl-terminated SAM surfaces. Methyl SAM: filled squares, solid regression line ($r^2 = 0.823$). Hydroxyl SAM: filled circles, broken regression line ($r^2 = 0.762$).

0.9 U/mg for the methyl- and hydroxyl-terminated SAM surfaces, respectively. The specific activity of CALB in solution was 4.2 U/mg, so adsorbing CALB to the methyl-terminated SAM surface appeared to improve its activity. However, given the level of uncertainty in measuring the reaction rate with CALB immobilized, a more conservative interpretation is that CALB did not suffer activity loss with methyl-terminated SAM immobilization.

CALB is not known to exhibit interfacial activation, not having a lid-like polypeptide loop in proximity to the active site that moves in response to hydrophobic surface abutment [23]. Blanco and coworkers [25] found that CALB adsorption to hydrophobic silica results in substantial pNPP hydrolytic activity loss. Reetz and coworkers [27] demonstrated only 52% specific activity retention with CALB immobilized in hydrophobic SiO_2 sol–gels. Based on a calculation of catalytically active CALB immobilized on polypropylene, determined by active site titration, only about 26% of the adsorbed lipase retains activity (7.8 mg of active CALB out of the 30 mg of CALB immobilized per g of support) [28]. Less than 50% of CALB adsorbed to methyl methacrylate resin retains activity [16]. Thus the observation that CALB immobilized on a methyl-terminated SAM surface had as great or greater activity than the enzyme in solution was somewhat unexpected. Activity retention differences between CALB on a hydrophobic resin or porous silica and that observed here with CALB on a flat surface may in part be ascribed to the lack of substrate internal diffusion limitations in the latter situation, or they may reflect an archi-

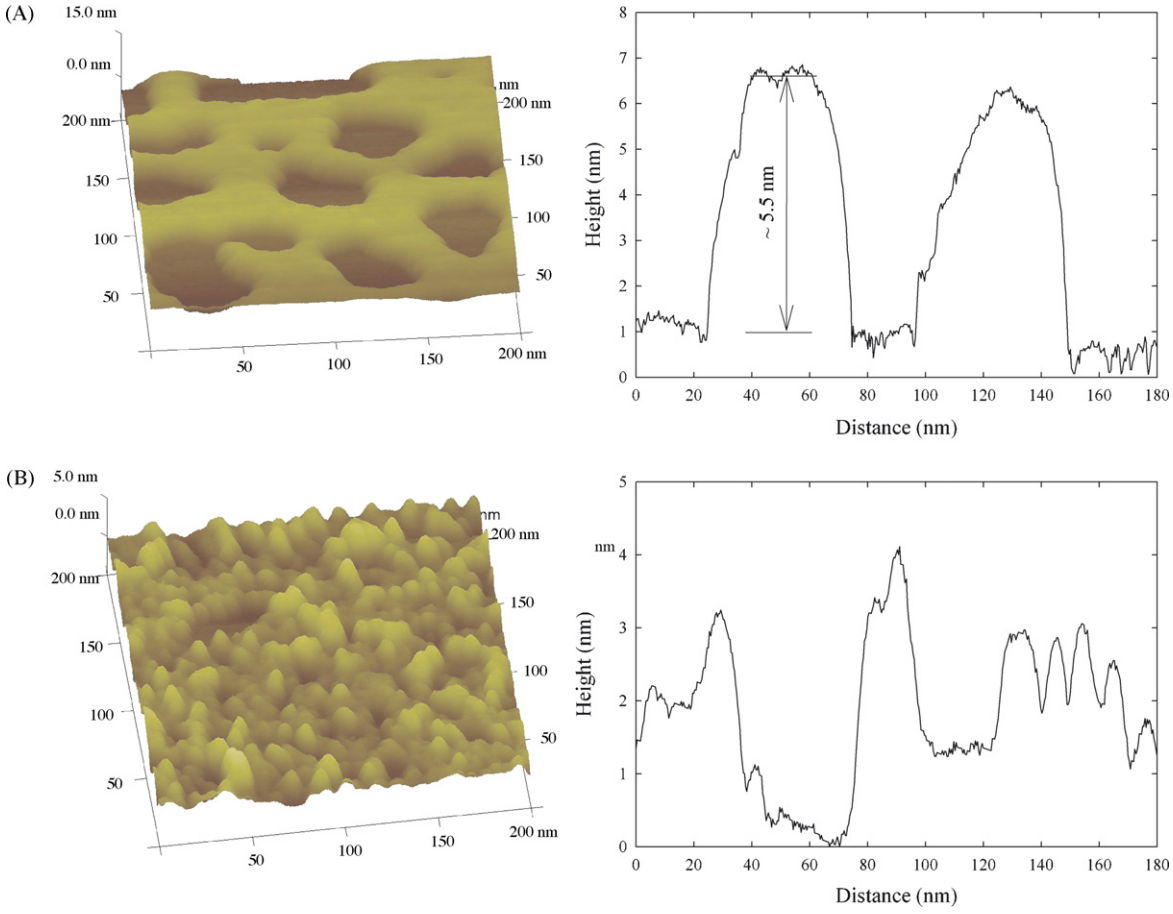

Fig. 3. AFM images of CALB on (A) methyl- and (B) hydroxyl-terminated SAM surfaces. The scan size was 200 nm × 200 nm. The accompanying section image analyses were mid-*y*-axis line scans. Each section analysis *z*-axis minimum was assigned a value of zero and all other height values are relative to this minimum. Images were not subjected to global flattening or filtering.

tectural arrangement of CALB on a methyl-terminated SAM surface that does not result in adverse structural changes to the enzyme.

Disordered CALB aggregation alone does not result in hydrolytic activity loss. Cross-linked enzyme aggregates of CALB retained high pNPP hydrolysis rates, some even showing enhanced activity compared to native enzyme, depending on the glutaraldehyde treatment conditions [29]. The substantial loss of CALB hydrolytic activity observed upon adsorption to a hydroxyl-terminated SAM surface therefore must be attributed to an interfacial influence.

3.4. AFM imaging of adsorbed CALB

Although CALB adsorbs to the same extent on hydroxyl- and methyl-terminated SAM surfaces, apparently with near monolayer coverage (based on the amount of protein absorbed and calculated surface area), the actual arrangement of the enzyme on the two surfaces may be very different. This could account for the large lipase activity differences between the two surfaces. AFM microscopy was employed to visualize CALB on hydroxyl- and methyl-terminated SAM surfaces. CALB (450 μg/mL) was adsorbed from buffer solution onto atomically flat SAM surfaces and then briefly dried under a stream of N_2. A 10 min incubation period was selected based on the QCM-D observation (Fig. 1) that coverage was not complete at this point and, therefore, portions of the underlying SAM surface could be reached with the AFM probe.

Fig. 3 shows that on either surface monolayers of CALB were formed, although their appearance was strikingly different. On the methyl-terminated SAM, CALB molecules were uniformly oriented with 5.5 nm peak heights. Even at very low concentrations (4.5 μg/mL), CALB formed clusters of uniform height (not shown) although native CALB does not form aggregates in solution [30]. The 5.5 nm height found for CALB on a methyl-terminated SAM (Fig. 3) was consistent with the CALB crystallographic structure. Its longest intramolecular distance is 5.74 nm (Thr316 to Pro268). The observation of 5.5 nm high CALB molecules suggests its longest axis is oriented perpendicular to the surface. CALB on a hydroxyl-terminated SAM displayed slightly lower peak heights, 2–4 nm high, which may reflect a flattening or distortion of the molecules on the surface (Fig. 3B). Such surface-induced structural changes in proteins are common [31], but there has been no such finding reported in the literature regarding CALB. The aggregation state of CALB on methyl methacrylate resin has been suggested recently to play a role in the activity of CALB [16]. AFM imaging indicated that adsorption geometry of CALB, as influenced by surface hydrophobicity, may have an important impact on catalytic efficacy.

4. Conclusions

Consistent with the general finding that CALB immobilization on hydrophobic supports yields the best enzyme activity, the present work demonstrated that the hydrophobic surface of a methyl-terminated SAM allowed the lipase to adsorb with full retention of specific activity. On this surface the protein assumed a highly packed monolayer of uniform molecular arrangement. CALB adsorption onto a hydrophilic, hydroxyl-terminated SAM led to a substantially lower specific activity and disordered protein arrangement. Interfacial interactions between the support and enzyme were responsible for the different outcomes. Although CALB is not subject to interfacial activation, it does have an immobilization surface preference.

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