Orientating lipase molecules through surface chemical control for enhanced activity: A QCM-D and ToF-SIMS investigation

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ABSTRACT

Bio-active materials consisting of lipase encapsulated within porous silica particles were engineered to control the adsorption kinetics and molecular orientation of lipase, which play critical roles in the digestion kinetics of triglycerides. The adsorption kinetics of *Candida antartica* lipase A (CalA) was monitored using quartz crystal microbalance with dissipation (QCM-D) and controlled by altering the hydrophobicity of a silica binding support. The extent of adsorption was 2-fold greater when CalA was adsorbed onto hydrophobic silica compared to hydrophilic silica. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) fragmentation patterns, in conjunction with multivariate statistics, demonstrated enhanced exposure of the lipase's catalytic domain, specifically the histidine group responsible for activity, when CalA was adsorbed on hydrophilic silica. Consequently, lipid digestion kinetics were enhanced when CalA was loaded in hydrophilic porous silica particles, *i.e.*, a 2-fold increase in the pseudo-first-order rate constant for digestion when compared to free lipase. In contrast, digestion kinetics were inhibited when CalA was hosted in hydrophobic porous silica, *i.e.*, a 5-fold decrease in pseudo-first-order rate constant for digestion when compared to free lipase. These findings provide valuable insights into the mechanism of lipase action which can be exploited to develop smarter food and drug delivery systems consisting of porous lipid-based materials.

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1. Introduction

Lipases are interfacially active enzymes that naturally catalyze the hydrolysis of triglycerides to form glycerol and free fatty acids. The rate and extent of lipid digestion can be manipulated by immobilizing lipase molecules within porous materials [1]. Upon physical adsorption at an interface, a lipase undergoes a conformational change whereby the alpha helix lid domain covering the active site opens to allow substrate molecules access to the non-polar catalytic domains [2]. This physical equilibrium can be described by Eq. (1) whereby the process of [Interface-Lipase]* complex formation, where lipase is in its active conformation, is described by the attachment and detachment rate constants, k_1 and k_{-1}, respectively [3].

$$Interface + Lipase \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Interface - Lipase]^* \quad (1)$$
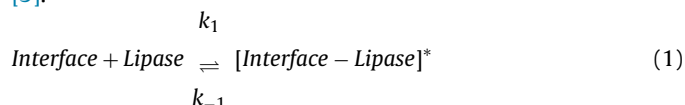
The formation of a [Interface-Lipase]* complex enables lipase molecules to catalyse the hydrolysis of triglycerides into free fatty acids and monoglycerides, or the esterification of digestion products into triglycerides (Eq. (2)) [4]. This chemical equilibrium can be described by hydrolysis and esterification rate constants, k_H and k_E, respectively [5].

$$[Interface - Lipase]^* + triglycerides \underset{k_E}{\overset{k_H}{\rightleftharpoons}}$$

$$fattyacids + monoglycerides + [Interface - Lipase]^* \quad (2)$$
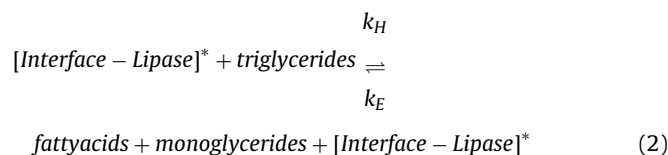
The unique property known as 'interfacial activation' sets lipases apart from other enzymes [6], and has led to their immobilization in a wide range of mesoporous materials for enhanced catalysis [7], *e.g.*, mesoporous silica [8–10]. Despite this, there is little known about the role of material characteristics on lipase-lipid interaction, the exact mechanism by which interfacial activation occurs and the parameters that control the hydrolysis equilibrium. Recently, we demonstrated the ability to control pancreatic lipase-mediated digestion of lipid loaded in porous silica particles [11]. An optimal pore size and structure, along with lipid coverage existed for maximum lipase activity [12]. Lipid adsorbed at a submonolayer coverage in hydrophilic porous silica was presented to lipase in

a favorable orientation compared to hydrophobic porous silica, thereby enhancing lipid digestion kinetics [13].

One area in the literature with a high level of conjecture, however, is the role of surface chemistry on interfacial activation of lipase molecules. Numerous forms of lipase have demonstrated enhanced hydrolytic activity when immobilized in porous hydrophobic supports [1,14–16]. However, previous studies have also shown optimal activity in materials with intermediate surface wettability [17] and poor activity at high contact angles [18–20]. This was attributed to stronger (hydrophobic) interactions between the enzyme's nonpolar domains and methyl groups at the interfaces, causing one of the following hypothesised mechanisms: (i) distortion of the active site and thereby, protein denaturation [21,22]; (ii) differences in lipase orientation reduces accessibility for the substrate due to greater exposure of the enzyme's polar, noncatalytic domains toward the aqueous phase [19,23]; or (iii) reduced lateral mobility of the adsorbed enzyme leads to shorter exposure time of the active site toward the aqueous solution [24–26].

In this study, we deconvolute the relationship between lipase adsorption kinetics and orientation on lipolytic activity by altering the surface chemistry of carrier particles. A common industrial lipase, *Candida antarctica* lipase A (CalA), was adsorbed in hydrophilic porous silica particles and hydrophobic, methylated porous silica particles to investigate the role of surface chemistry on lipase-mediated digestion. Adsorption kinetics on the two surfaces were monitored using quartz crystal microbalance with dissipation (QCM-D), to establish the affinity CalA has for both surfaces through changes in vibrational frequency and dissipation. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to compare differences in spectrometric fragmentation patterns that can indicate subtle changes in lipase conformation or orientation at each surface [13,27–29]. In doing so, the critical parameters that effect the rate and extent of lipolysis were determined. This will contribute to the optimisation of lipid digestion in porous lipid drug carriers for enhanced fat and drug solubilisation and absorption [17,30–33].

2. Materials and methods

2.1. Materials

C. antarctica lipase A (CalA) was purchased from Sigma-Aldrich (Australia). Hydrophilic porous silica particles, PS-1 (Aerosil 200, surface area $200\,m^2/g$) and hydrophobic porous silica particles, PS-2 (Aerosil R812, surface area $220\,m^2/g$, hydrophobized with hexamethyldisilazane (HMDS) by manufacturer) were supplied by Evonik (Essen, Germany). Tributyrin (TB) was supplied by Acros Organics, ThermoFisher Scientific (Scoresby, Australia). Chlorotrimethylsiliane, phosphate buffered saline (PBS) tablets, tris buffered saline (TBS) sodium hydroxide pellets, Bradford reagent and cyclohexane were all purchased from Sigma-Aldrich (Australia). QCM-D sensor crystals (5 MHz), reactively sputter-coated with 50 nm silicon dioxide, were purchased from Q-Sense (Gothenburg, Sweden). All chemicals were of analytic grade and used as received. High purity (Milli-Q) water was used throughout the study.

2.2. Quartz crystal microbalance with dissipation (QCM-D) measurements

2.2.1. QCM-D instrumental

Quartz crystal microbalance with dissipation (QCM-D) measurements were performed on silicon dioxide coated QCM-D crystals (25 mm, 5 MHz) using a Q-Sense E4 system (Q-Sense, Inc.,

Biolin Scientific AB, Sweden) and adapted from Laszlo & Evans [34]. The QCM-D technique, detailed elsewhere [35], probes the amount of adsorbed mass (including coupled water) through changes in the vibrational frequency, *f*, and the degree of frictional (viscous) losses in the adsorbed layer through changes in dissipation, *D*.

The Sauerbrey equation relates measured frequency shift (Δf) with the change in adsorbed mass per unit area (Δm) [36]:

$$\Delta m = -\frac{C\Delta f}{n} \tag{3}$$

where *C* is the mass sensitivity constant ($17.7\,ng\,cm^{-2}\,Hz^{-1}$ for 5 MHz crystals) and *n* is the overtone number. The Sauerbrey equation can only be used to confidently estimate Δm for rigid layers, *i.e.,* $\Delta D \le 10^{-6}$ [35]. The mass calculated from the frequency change is only an approximation because the frequency change is due to the adsorbed material and any trapped or associated water molecules.

2.2.2. Preparation of hydrophobic SiO_2 crystals

Silica coated QCM-D crystals were hydrophobized by immersion in a chlorotrimethylsilane (20 mM) in cyclohexane solution for 12 h at ambient temperature (25 °C), followed by rinsing with Milli-Q water prior to measurements. Sessile drop measurements were performed to determine contact angles of the QCM-D crystals. In all cases, the contact angles were greater than 90°.

2.2.3. Lipase adsorption kinetics by QCM-D

Silica coated crystals (5 MHz) were mounted into the QCM-D unit and flushed with PBS solution (25 mM, pH 7.5) to establish baseline values. The crystal and solution chamber temperature was maintained at 25 ± 0.1 °C. CalA solutions (0.6, 1.5, 3.0, 15 and 30 mM) in PBS (25 mM, pH 7.5) were slowly introduced into the QCM-D cell (0.1 mL min⁻¹), allowing lipase to adsorb onto the crystal surface under flow conditions for approximately 10 min. In doing so, the processes of lipase adsorption and surface adlayer changes were followed *in situ* while continuously exposing the surface with different solutions. The cell was flushed with buffer to determine equilibrated changes in crystal frequency and dissipation. Measurement data for *f* and *D* were acquired at several harmonics simultaneously. All QCM-D experiments were repeated and demonstrated good reproducibility.

Adsorption versus concentration profiles from QCM-D data were analysed by fitting to the Langmuir adsorption isotherm (Eq. (4)). The maximum surface coverage of CalA, Γ_{max}, and Langmuir binding constant, K_L, were determined.

$$\Gamma_{CalA} = \frac{\Gamma_{max}K_L[CalA]}{1 + K_L[CalA]} \tag{4}$$

where Γ_{CalA} is the surface coverage of CalA and [CalA] is the initial concentration of CalA in the bulk solution. Surface coverage values were derived from adsorbed mass changes that were obtained using the Sauerbrey equation. The Langmuir binding constant, K_L, is equal to the inverse of the equilibrium dissociation constant, K_D, which is the concentration of CalA required for 50% surface coverage (Eq. (5)) [37].

$$[CalA] = K_L^{-1}\frac{\vartheta}{1 - \vartheta} \tag{5}$$

where K_L^{-1} is equal to K_D and ϑ is the relative CalA surface coverage (given by $\Gamma_{CalA}/\Gamma_{max}$).

The Langmuir model makes the following assumptions regarding the adsorption mechanism: (i) all adsorption sites are equivalent, (ii) there are no interactions between adsorbed molecules, (iii) all adsorption occurs through the same mechanism, and (iv) a monolayer is formed at maximum adsorption [38]. We apply this model understanding the limitations associated with correlating CalA adsorption to the Langmuir binding constant. Thus,