Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Atsushi KOBAYASHI, Yukari SATO & Fumio MIZUTANI (2001) Adsorption Properties and Activities of Lipase on a Gold Substrate Modified by Self-assembled Monolayers, Bioscience, Biotechnology, and Biochemistry, 65:11, 2392-2396, DOI: 10.1271/bbb.65.2392

To link to this article: http://dx.doi.org/10.1271/bbb.65.2392

Biosci. Biotechnol. Biochem., **65** (11), 2392–2396, 2001

Adsorption Properties and Activities of Lipase on a Gold Substrate Modified by Self-assembled Monolayers

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Received March 30, 2001; Accepted July 12, 2001

The adsorption properties, amount and specific activity of lipase D from *Rhizopus delemar* were investigated by employing a gold substrate modified with seven kinds of thiol monolayer. Quartz crystal microbalance measurements revealed that the amount of the enzyme adsorbed to the hydrophobic monolayers (*e.g.* benzenethiol) was much higher than that to the hydrophilic monolayers (*e.g.* 3-mercaptopropanoic acid). In contrast, lipase D adsorbed to the hydrophilic, 2-amino-1-ethanethiol monolayer showed the highest specific activity, the value being 300-fold higher than for the same enzyme dissolved in an aqueous medium.

Key words: self-assembled monolayer; lipase; interfacial activation; quartz crystal microbalance

Lipases (glycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes which have considerable physiological significance and industrial potential.[1] The interaction of lipases with insoluble substrates does not conform to Michaelis-Menten kinetics, but instead involves two distinct steps: adsorption to an oil-water interface and subsequent lipolysis. The catalytic activity of the enzyme increases dramatically at the interface, this phenomenon being known as interfacial activation. The fact was initially established in 1958 by Sarda and Desnuelle,[2] and the activation of lipase caused by immobilization on a solid support has been reported in several studies.[3-6] Crystallographic studies have shown that interfacial activation was associated with a conformational change.[7-9] However, the mechanism for triggering the opening of the lid has been unclear, although many works have been done.[10,11]

To elucidate the triggering mechanism, lipase D from *Rhizopus delemar* with a molecular mass of 44 kDa was selected and used in this work. Lipase D is an attractive model of lipase for the type of study described in this work, since it is a microbial lipase without cofactors, unlike mammalian lipase, and is well-characterized and commercially available in pure form.[12,13]

To identify the type of interface that can activate lipase D, the amount and activity of lipase adsorbed to various modified surfaces were measured. One of the major methods for understanding interfacial activation is to use the lipid monolayer at an air/water interface,[14,15] because this mimics the natural substrates of lipases. However, there are some limitations, *i.e.*, the availability of lipids and the effect of ionic strength on the lipid lateral structure.

The self-assembly technique is an ideal method for creating a two-dimensional membrane. Extensive work has indicated that alkanethiols can be adsorbed to a metal surface and produce a stable and highly ordered structure.[16] Alkanethiols attached to gold electrodes have been used as anchors to immobilize biological molecules in order to construct both enzyme biosensors and immunosensors.[17,18] Thus, self-assembled monolayers would be a powerful tool for simultaneously analyzing the adsorption behaviour and activity of lipase.

We applied quartz crystal microbalance (QCM) measurement to evaluate the amount of adsorbed lipase. QCM having been successfully used in situ to monitor the binding of proteins in an aqueous solution.[19-21]

We report the adsorption properties, activities, and stability of lipase D on seven kinds of self-assembled monolayer (SAM) in this paper.

Materials and Methods

Chemicals. Lipase D (from *Rhizopus delemar*) was purchased from Seikagaku Kogyo. Its optimum temperature, pH, and pI were 40°C, 5.6, and 8.2, respectively.[22,23] 1-Mercaptododecane (C12), 1-mercap-

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Abbreviations: SAM, self-assembled monolayer; QCM, quartz crystal microbalance; THF, tetrahydrofuran; BNT, benzenethiol; C12, 1-mercaptododecane; C4, 1-mercaptobutane; 4HBT, 4-hydroxybenzenethiol; 2AET, 2-aminoethanethiol; 3MPA, 3-mercaptopropanoic acid; C4OH, 4-hydroxy-1-mercaptobutane; pNPA, *p*-nitrophenyl acetate

tobutane (C4), benzenethiol (BET), 4-hydroxybenzenethiol (4HBT), 2-amino-1-ethanethiol (2AET), 3-mercaptopropanoic acid (3MPA), and 4-hydroxy-1-mercaptobutane (C4OH) were purchased from Sigma. *p*-Nitrophenyl acetate was also purchased from Sigma as a substrate of lipase. All these chemicals were used without further purification.

Adsorption of the thiol compounds. Prior to use, the gold substrates and quartz crystals were cleaned by immersing in a piranha solution of H_2SO_4/H_2O_2 (3:1). The modification was performed by immersing a gold substrate (vacuum-deposited gold on glass, thickness: 100 nm) in an ethanol solution of a thiol (10 mM) for 2 hr. After the modification had been achieved, the substrate was thoroughly washed in pure ethanol.

Measurement of the adsorption of lipase D to SAMs. The amount of lipase adsorbed to each thiol monolayer on gold was detected out by QCM. The frequency decrease of the QCM indicates the mass increase corresponding to the addition of lipase from the medium. Experiments were carried out at 20°C. A 10 MHz, AT-cut quartz crystal with deposited gold on both sides (1.6 cm^2 in area, Hokuto Denko) was used for the QCM measurements. The frequency change was monitored by an HQ-101B EQCM controller (Hokuto Denko) attached to a personal computer. In the case of protein adsorption in an aqueous solution, the calibration showed that a frequency decrease ($-\Delta F$) of 1 Hz corresponded to a mass increase (Δm) of 4.4 ng cm^{-2}.

Enzyme activity assay. The enzyme reaction was performed in a cuvette (1.0×4.0 cm). Lipase was adsorbed by immersing the gold plate (2.4 cm^2 in area) into a 10 mM acetate buffer (pH 5.6) with 3.3 μg/ml of lipase for 5 hr. The gold plate was removed from the solution and dried in fresh air.

A substrate solution (0.25 M, 0.05 ml) containing 1.4% of ethanol was added to 50 ml of a 0.01 M acetate buffer solution (pH 5.6), and the whole was incubated at 40°C. The lipase-adsorbed SAM-modified gold substrate was then put into the cuvette, and the enzyme reaction was monitored with a UV-160 instrument (Shimadz). The amount of *p*-nitrophenol was determined by measuring the absorbance of the solvent layer at 400 nm. Each enzyme assay cycle was performed for 30 min, before the next cycle proceeded.

Results and Discussion

Effect of the initial lipase content on the adsorption to the monolayer

Most lipases are highly soluble proteins that show esterase activity even in a fully aqueous medium; that is, they remain water-soluble even when partially exposing the very large hydrophobic pocket at their catalytic center. We first considerd that the hydrophobic catalytic center of lipase D could be directsd to the hydrophobic surface and investigated the surface property-dependent adsorption of lipase D by the QCM method.

The amounts of lipase D adsorbed to both the BNT-modified gold surface and unmodified surface are summarized in Table 1. The size of lipase D was estimated from the X-ray crystallographic data to be ca. $5\times5\times5$ nm^3.[9] If lipase D was compactly adsorbed to the monolayer, then the theoretical Δm_{max} value was calculated to be ca. 3.7×10^{-7} g cm^{-2}. The adsorption of lipase D to bare gold depended on the lipase D concentration. At the initial lipase D concentration of 33 μg/ml, the amount adsorbed was 3.5×10^{-7} g/cm^2, which is similar to the theoretical saturation value for monolayer adsorption. On the other hand, the adsorption of lipase D to the BNT monolayer was saturated at an initial lipase D concentration of 3.3 μg/ml. These results indicate that the BNT monolayer was preferred for the adsorption of lipase and the non-specific adsorption occurred on the bare gold surface.

Stability of adsorbed lipase D

The stability of lipase D adsorbed to various monolayers was next determined. Figure 1 shows the residual activity of lipase D adsorbed to hydrophobic

Table 1. Effect of Initial Lipase Concentration on Adsorbed Lipase

Initial conc. (mg ml^{-1})	Lipase content (10^{-7} g cm^{-2})	
	BNT	Bare gold
3.3	5.1±0.9	1.4±0.7
33	3.5±0.6	3.5±0.2

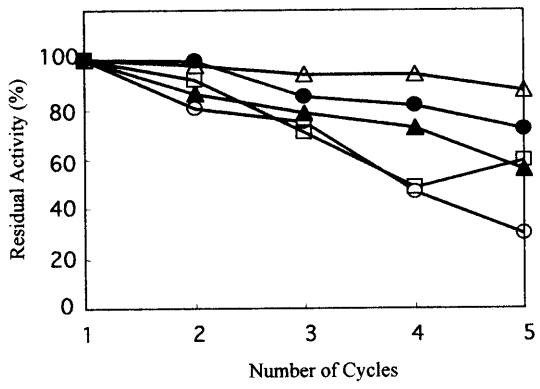

Fig. 1. Stability of Lipase D Adsorbed to Hydrophobic Self-assembled Monolayers.

● BNT, benzenethiol; ○ bare gold; △ C12, 1-mercaptoundeane; ▲ C4, 1-mercatobutane; □ 4HBT, 4-hydroxy-benzenethiol.

monolayers for the hydrolysis of pNPA. The lipase D-adsorbed C12 monolayer retained 90% of the initial enzyme activity after 5 successive enzyme activity measurements and showed the highest residual activity among the monolayers. The activity of the lipase D-adsorbed C4 and 4HBT monolayers gradually declined during successive measurements, and the residual activity was 60% of the initial level after five measurements. The weak hydrophobic interaction between lipase D and the C4 and 4HBT monolayers resulted in the progressive release of lipase D from the monolayers because the hydrophobicity was lower than that of the C12 and BNT monolayers.[16]

Figure 2 shows the activity of lipase D adsorbed to the hydrophilic monolayers for the hydrolysis of pNPA. The activity dropped dramatically during successive measurements, mainly due to the adsorption of lipase D by non-specific binding, weak electrostatic interaction and/or hydrogen bonding. It remains unconfirmed whether the decrease on enzyme activity was caused by inactivation or desorption.

Monolayer specificity for the adsorption of lipase D

The adsorption properties of lipase D to bare gold and to the seven kinds of self-assembled monolayer were evaluated by QCM. The highest frequency change was observed for the BNT monolayer among the seven kinds of SAM, and being equivalent to an adsorbed amount of lipase D of 12.0×10^{-7} g to the BNT monolayer (Fig. 3).

The BNT and C12 monolayers were highly hydrophobic, and lipase D prefers long-chain esters. The amounts of lipase D adsorbed to the C12 and BNT monolayers were the highest among all the seven monolayer systems studied. It is presumed that the high affinity between these hydrophilic monolayers and lipase D was expressed by interfacial activation.[3,18,24] The amounts of lipase D adsorbed to the other hydrophilic monolayers were relatively small, and there was no significant difference among them.

It has been reported that the 3MPA monolayer was anionic in an aqueous solution of pH $\gg$ 5.6.[25] Lipase D was cationic under our experimental conditions,[22] and the affinity between the 3MPA monolayer and lipase D was low. Although the affinity between the 3MPA monolayer and lipase D was caused by electrostatic interaction, lipase D adsorbed to the 3MPA monolayer was expected to be desorbed from the 3MPA monolayer because the carboxyl group was one of the products of the lipase D-hydrolytic reaction. Since a substrate needs to be released from the active site of an enzyme for hydrolysis, a low level of adsorption of lipase to the 3MPA monolayer was observed.

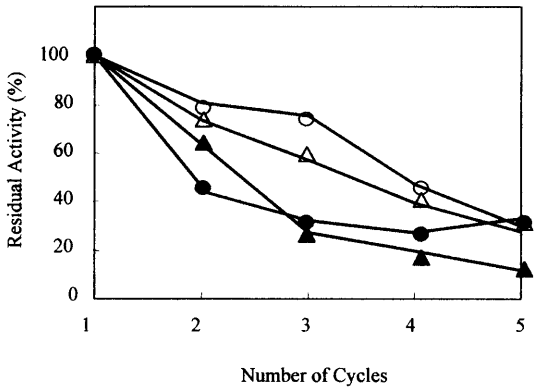

Fig. 2. Stability of Lipase D Adsorbed to Hydrophilic Self-assembled Monolayers.

○ Bare gold; ● C4OH, 4-hydroxy-1-butanethiol; △ 3MPA, 3-mercaptopropionic acid; ▲ 2AET, 2-amino-1-ethanethiol.

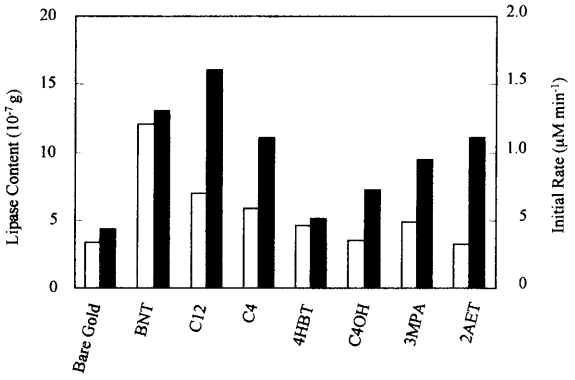

Fig. 3. Amount of Lipase Adsorbed to a Gold Substrate Modified with Self-assembled Monolayers and Activity of Adsorbed Lipase.

Filled bar, initial rate; unfilled bar, lipase content. The lipase-adsorbed area on the gold substrate was 2.4 cm^2.

Table 2. Specific Activity of Lipase Adsorbed to SAMs

	Specific activity (mmol min^{-1} (g of lipase)$^{-1}$)
Solubilized lipase	0.042
BNT	4.6
4HBT	3.9
C4OH	7.2
3MPA	7.0
2AET	12

Activity of adsorbed lipase D

We determined the lipase activity at the various interfaces by using pNPA as a substrate. The specific activity (mM/min/(g of protein)$^{-1}$) of the adsorbed lipase D is summarized in Table 2. Any type of interface activated lipase D, unlike the case of lipase D in a solution. The activity of lipase D adsorbed to each kind of interface was at least 100-fold greater than that of soluble lipase D. Lipase D adsorbed to the 2AET monolayer showed the highest specific activity

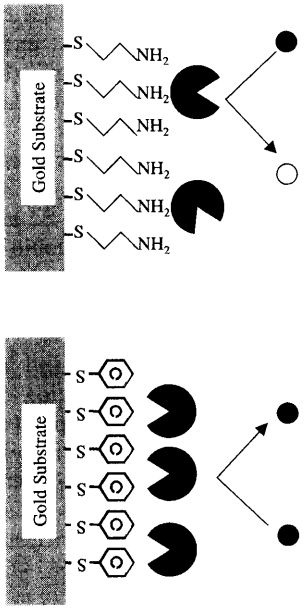

Fig. 4. Proposed Mechanism for Lipase Activation at the Interface.

Lipase is adsorbed to the BNT monolayer strongly and densely because the BNT monolayer is hydrophobic like the substrate. It is difficult for the substrate to access the active site of lipase. On the other hand, lipase was adsorbed to the 2AET monolayer weakly and sparsely. Thus, the substrate could easily enter the active site of lipase on the 2AET monolayer. Filled circle, substrate; unfilled circle, product.

among the various SAMs.

Cajal *et al.* have reported that a lipase from the fungus, *Thermomyces lanuginosa* (TlL), was activated in the presence of large anionic vesicles of 100 nm diameter during the hydrolysis of *p*-nitrophenyl butyrate.[26] They also reported that this interfacial activation was not supported by zwitterionic vesicles. Their proposed model for adsorption and activation is consistent with the X-ray structure of TL in a complex with inhibitor.[27] Certain cationic residues located in the hinge regions on both sides of the lid contributed to opening of the lid and to stabilization with the lid opened.

In the case of lipase D, two anionic residues were located in the lid region.[28] It was assumed that the electrostatic interaction between the 2AET monolayer and lid residues contributed to stabilization in the open conformation.

Orientation of lipase D at the interface

Lipase D at an interface is presumed to be oriented so that the exposed large hydrophobic concavity around the active site that must interact with the substrate is exposed to the interface with its adsorption (Fig. 4 left).[4,7] Our results with various hydrophilic and hydrophobic SAMs show that any kind of interface activated lipase D (Table 2). Moreover, lipase D adsorbed to the 2AET monolayer was more active and showed higher specific activity than when adsorbed to the other hydrophilic monolayers. This result suggests that the orientation of adsorbed lipase depended on the properties of the interface (Fig. 4). The soluble substrate, pNPA, might have created difficulty for accessing the active site, because of the high density of lipase D at a hydrophobic interface and because of the interface-facing orientation of the active site (as shown in Fig. 4 left). On the contrary, non-specific adsorption to the hydrophilic 2AET monolayer rendered the active site exposed to the bulk solution (as shown in Fig. 4 right), and the specific activity was higher than that with the hydrophobic monolayers.

The surface hydrophobicity of a solid support is an important criterion for lipase adsorption. However, the introduction of a hydrophilic group to the surface would be required to activate the adsorbed lipase. As a consequence, a combination of both hydrophobic and hydrophilic properties at the interface, *i.e.* mixed monolayers, might be an appropriate solution for rigid adsorption, long-term stability, high activation, and the most suitable orientation of lipase.

Acknowledgment

We thank Dr. K. V. Gobi for useful discussions.

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