# Observation of Phospholipase $A_2$ Activity Towards the Hydrolysis of Phospholipid Langmuir–Blodgett Films Deposited on a Quartz-crystal Microbalance

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Catalytic hydrolysis of phospholipid Langmuir–Blodgett (LB) films deposited on a quartz-crystal microbalance (QCM) by phospholipase $A_2$ was followed by observing frequency changes of the QCM in an aqueous solution.

Phospholipase $A_2$ ($PLA_2$) is a small, water-soluble enzyme that catalyses the specific hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides.[1] $PLA_2$ can catalyse effectively aggregated lipid molecules such as micellar,[2] vesicular[3] and monolayer states[4] compared with monomeric substrates.[5] This indictes that the binding (selective adsorption) of $PLA_2$ to lipid interfaces is also important as well as the catalytic hydrolysis step. The former methodologies, such as pH-stat titrations for micellar or vesicular systems[2,3] and fluorescence spectra or radioisotope techniques for monolayer systems,[4] have some difficulties in quantitative kinetic analyses for both the binding step of $PLA_2$ to lipid interfaces and the catalytic hydrolysis step of lipid molecules.

In this communication, we propose a new methodology to investigate the $PLA_2$ hydrolytic activity, using reactions between Langmuir–Blodgett (LB) films of phospholipids deposited on a quartz-crystal microbalance (QCM) and $PLA_2$ in an aqueous solution. QCMs are known to provide very sensitive mass measuring devices because the resonance frequency decreases upon the increase of a given mass on the QCM electrode in a nanogram level.[6–10]. We can expect to detect dirctly the amount of $PLA_2$ binding to the lipid membrane (mass increase) and then the catalytic hydrolysis of phospholipid LB films (mass decrease) on a QCM from frequency changes. We have reported, by using the QCM method, that a deposition process of LB films from the air–water interface,[7] a swelling or flaking behaviour of LB films in the aqueous phase or at the air–water interface,[8,9] and a phase transition behaviour (a fluidity change) of LB films on the QCM.[10]

The QCM employed is a commerically available 9 MHz, AT-cut quartz (diameter: 8 mm, Au electrode area: 15.9 $mm^2$),[7–10] one side of which is covered by a rubber seal in order to avoid an electric short-circuit in aqueous solutions. The QCM was connected to a handmade oscillator designed to drive the quartz at its resonance frequency in aqueous solutions.[7–10] The QCM was driven at 5 V DC, and the frequency of the vibrating quartz was followed by a universal frequency counter (Iwatsu Co., Tokyo, model SC 7201) attached to the microcomputer system (NEC Co., Tokyo, model PC 9801). Calibration showed that a frequency decrease of 1 Hz corresponded to a mass increase of 1.05 ng of the QCM electrode.[7–10]

Eight layers of dipalmitoyl(3-*sn*-phosphatidyl)ethanolamine (DPPE) monolayer were deposited on one side of a QCM at a surface pressure of 45 mN $m^{-1}$ (pH 5, Milli-Q water) using the LB technique. It was confirmed by observing the frequency decrease of the QCM that the mass deposited

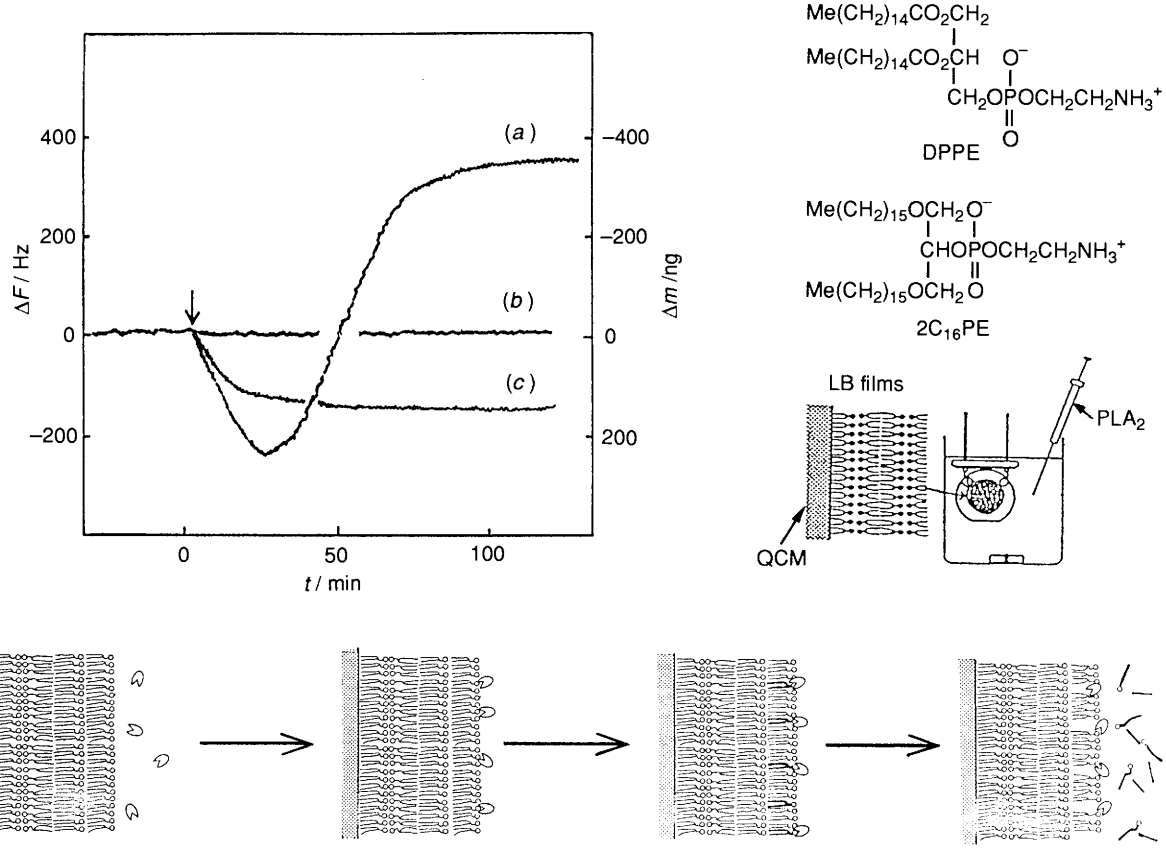


**Fig. 1** Typical frequency changes on the phospholipid LB film (eight layers) deposited on a QCM responding to the addition (marked by the arrow) of phospholipase (14.5 unit) in an aqueous solution (pH 8.0, 10 mmol $dm^{-3}$ TRIS buffer, 37 °C) the (*a*) DPPE membranes + $PLA_2$ (porcine pancreas); (*b*) $2C_{16}PE$ membranes + $PLA_2$ (porcine pancreas); (*c*) DPPE membranes + PLC (*Baccilus cereus*)

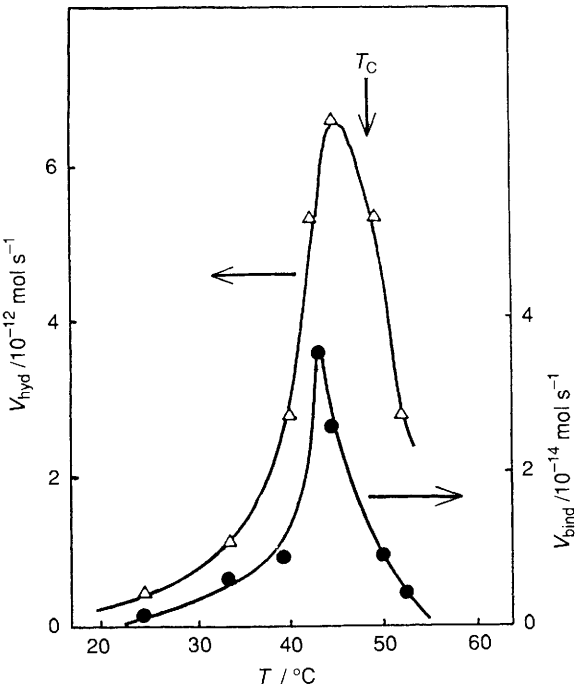


**Fig. 2** Temperature dependencies of binding ($V_{bind}$) and hydrolysis rates ($V_{hya}$) of DPPE LB films catalysed by $PLA_2$ (from porcine pancreas, 14.5 unit, pH 8.0, 10 mmol $dm^{-3}$ TRIS buffer)

on the QCM plate (360 ± 10 ng, corresponding to a frequency decrease of 343 ± 10 Hz) was consistent with the theoretical mass of eight layers of DPPE (358 ± 5 ng).

Fig. 1 [curve (*a*)] shows frequency changes of the DPPE LB film (eight layers) deposited QCM when 14.5 units of $PLA_2$ (from porcine pancreas, Sigma) was injected into the aqueous solution (pH 8.0, 10 mmol $dm^{-3}$ TRIS buffer, 37 °C) under mild stirring. Stirring did not affect the frequency measurements. The frequency of the QCM decreased by 230 Hz (corresponding to 242 ng of mass increase) in 25 min, then increased gradually, and reached an increase of 340 ± 10 Hz beyond the starting point, where a decrease in mass of 357 ± 10 ng is consistent with the mass of the originally deposited DPPE LB films (360 ± 10 ng). The first frequency decrease (mass increase) shows the binding (selective adsorption) of $PLA_2$ onto the DPPE LB films on the QCM. The second frequency increase (mass decrease) shows the dissolution of hydrolysed single-chain substrates (palmitic acid and lyso-PE) from the QCM plate, and the deposited DPPE LB films were completely hydrolysed within 2 h. We have confirmed that double-chain DPPE LB films are stable on the QCM plate, but the mixed LB films of DPPE with single-chain palmitic acid and lyso-PE (more than 20 mol%) are easily flaked from the QCM plate within 30 s in the aqueous phase. Thus, the second frequency-increasing step indicates the rate-limiting hydrolysis step of DPPE LB films catalysed by the bound $PLA_2$.

We have also carried out TLC–FID (thin-layer chromatography with a flame-ionization detector, Iatron Lab. Co., Tokyo, model TF-10) analyses of DPPE on the QCM and lyso-PE in the aqueous phase on a large scale: the amount of DPPE on the QCM hardly decreased during the first frequency increase and the second frequency decrease consisted with the increase of the concentration of lyso-PE in the aqueous phase.

The similar frequency changes shown in a curve a of Fig. 1 were also observed in the DPPE-deposited QCM responding to the addition of other $PLA_2$ from different origins, and the order of their activities were as follows: bee venom ≫ porcine pancreas = Naja Naja venom.

When the 1,3-ether type phospholipid $2C_{16}PE$ LB films were employed instead of DPPE LB films on the QCM, the frequency of the QCM did not change responding to the addition of $PLA_2$ in the aqueous phase [curve (*b*) in Fig. 1]. This indicates that $PLA_2$ does not recognize and certainly does not hydrolyse the 1,3-ether type phospholipid membranes. When phospholipase C (PLC, from *Baccilus cereus*, Sigma) was added to the DPPE LB films on the QCM instead of $PLA_2$, only the first frequency decrease (mass increase) was observed [curve (*c*) in Fig. 1] and the formation of 1,2-dipalmitoylglycerol on the QCM was confirmed by the TLC–FID method. PLC is known to hydrolyse the phosphate ester bonds between glycerol and the PE head-group, and to give double-chain 1,2-diacylglycerol as products. Thus, curve (*c*) in Fig. 1 shows only the binding step of PLC on the lipid membranes and the catalytic hydrolysis step was not observed as frequency changes. This is because the double-chain glycerols produced are stable and not flaked from the QCM plate.

Fig. 2 shows the temperature dependences of the binding (adsorption) and the hydrolysis rates of dimirlystoyl(3-*sn*-phosphatidyl)ethanolamine (DMPE) LB films (eight layers) catalysed by $PLA_2$. The adsorption rates of enzymes into lipid membranes were obtained from the initial slope of the frequency decrease (mass increase) shown in Fig. 1. The hydrolysis rates of DMPE molecules were obtained from a linear part of the following frequency increase (mass decrease), which indicates the release rate of the single-chain products from the QCM plate to the aqueous phase. Fig. 2 clearly indicates that both adsorption and hydrolysis rates drastically increased only near the phase transition temperature ($T_c$ = 49 °C) of DMPE LB films, compared with the solid (below $T_c$) and the liquid crystalline state (above $T_c$). The hydrolytic activity of $PLA_2$ toward micellar lipids gradually increased with temperatures in the range of 20–60 °C. This means that temperature dependencies shown in Fig. 2 are due to the physical state (phase transition) of lipid membranes, but not due to the change of enzymatic activity of $PLA_2$. Thus, $PLA_2$ selectively binds and hydrolyses the disturbed lipid membranes coexisting both solid and fluid liquid crystalline states near $T_c$, but not the well-packed solid state and the uniformly fluid liquid crystalline state.

In conclusion, the QCM method is useful to detect directly both the binding (selective adsorption) and hydrolysis steps of phospholipid membranes catalysed by $PLA_2$ in the aqueous phase. Detailed analyses of various phospholipase activities toward various phospholipid LB films using the QCM system are currently in progress in our laboratory.

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