Chinese Chemical Letters Vol. 14, No. 11, pp 1199 – 1202, 2003
http://www.imm.ac.cn/journal/ccl.html

"Lotus" Domain Formation by the Hydrolysis Reaction of Phospholipase D to Phospholipid Monolayer

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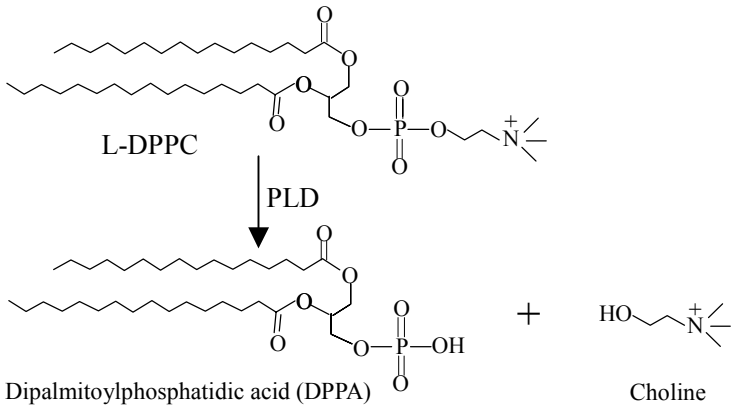
Abstract: Hydrolysis reaction of L-α-dipalmitoylphosphatidylcholine (L-DPPC) monolayer with phospholipase D (PLD) has been investigated by Brewster angle microscopy (BAM) combined with the film balance. It has been found that the L-DPPC domains were changed into the "lotus" structure by PLD. It suggests that the hydrolysis reaction is incomplete and the products together with the nonreacted materials undergo a molecular rearrangement at the interface.

Keywords: Brewster angle microscopy, phospholipase D, hydrolysis, phospholipid monolayer.

All chemical reactions in living cells depend on the impact of enzyme. One of the important enzymes in biological system is phospholipase D (PLD), which catalyzes the cleavage of the terminal phosphate ester bond at the polar head group of phosphatidylcholine to phosphatidic acid and a water-soluble choline as described **Figure 1**. This enzyme is widely present in plants, bacteria, and animals and involved in membrane formation, protein transport, regulation of mitosis and so on. The main product after hydrolysis, phosphatidic acid, is a second messenger and plays an important role in signal transduction pathways [1, 2].

Figure 1 Schematic representation of the hydrolysis of an L-DPPC monolayer by PLD



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Many different techniques have been employed to study the hydrolysis process of phospholipid monolayer or bilayer by phospholipase, such as surface X-ray diffraction [3], FT-IR spectroscopy [4], fluorescence microscopy [5] and radiolabeled phospholipid method [6]. And radiolabeling method or a two-step enzymatic measurement is the earliest and extensive method and many parameters of kinetics have been acquired by measuring the decrease in radioactivity of phospholipids. Direct observation of the hydrolysis process of enzymes on lipid monolayers is helpful in understanding the reaction mechanism.

Brewster angle microscopy (BAM) provides the opportunity to distinguish the individual domain change during the rearrangement of molecules in monolayers [7, 8]. In fact, BAM has also been proved to be a very useful technique to monitor the hydrolysis process of phospholipid monolayer by phospholipase and to get direct information on the molecular reorganization of lipid monolayers during the course of hydrolysis [9].

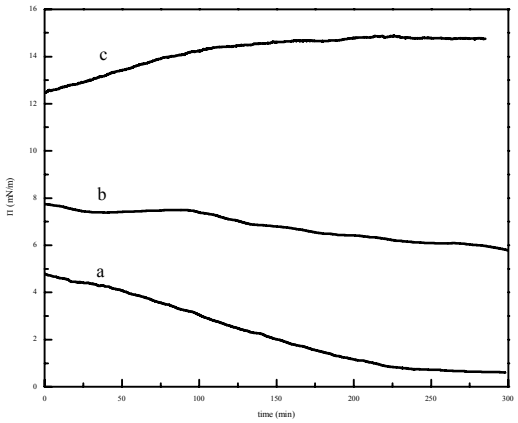
In this letter, we report to use BAM and the monomolecular film technique to investigate the hydrolysis process of L-DPPC by PLD. The hydrolysis mechanism has been studied by visualizing the morphological changes of L-DPPC condensed phase domains at different initial surface pressure.

The experimental setup used for the BAM and film balance measurement at the air/water interface is the same as described previously [10]. A commercial Brewster angle microscopy (Optrel, Germany) was mounted onto a computer-interfaced Langmuir trough (R&K, Germany) with a film balance. Cabbage PLD and L-α-dipalmitoyl-*sn*-glycero-3-phosphocholine (L-DPPC) were purchased from Sigma and used without further purification. Water was purified with a Millipore system. The phospholipid was dissolved in chloroform to prepare 1 mmol/L solution. The subphase was aqueous buffer solution with 40 mmol/L $CaCl_2$, 80 mmol/L CH_3COONa at pH 5.6. PLD was dissolved in the same buffer solution as used as a subphase. The monolayer was prepared by depositing drops of 1 mmol/L L-DPPC chloroform solution on the surface of the subphase at temperature of 20°C. After about 10 min the monolayer was compressed at 2.5×10^{-2} nm^2 per molecule per minute to a desired initial surface pressure and the enzyme (PLD) solution was then injected into the subphase and carefully stirred underneath the monolayer. At the same time, the π-t isotherm was recorded and the morphology changes of the monolayer were monitored by BAM. The trough area was maintained constant during hydrolysis.

The results of the measurements of surface pressure as a function of time are shown in **Figure 2**. It can be seen that when the initial surface pressure is at π = 4.8 and 7.8 mN/m respectively, the π-t curves drop down after the injection of the enzyme. This can be ascribed to the fact that the adsorption of enzyme and the hydrolysis take place simultaneously. One of the hydrolytic products, choline, is fully water-soluble and will leave the interface into the subphase. The other hydrolytic product, DPPA, has smaller headgroup than L-DPPC. And it will cause the decrease of the surface pressure. On the other hand, the adsorption of PLD into the lipid monolayer may result in the increase of π. At the lower initial surface pressure, the hydrolysis rate might be faster than the adsorption rate of enzyme. As a consequence, the surface pressure of the whole system is decreased. As the initial surface pressure is relatively higher, π = 12.5 mN/m, lipid monolayer remains at a condensed phase and the adsorption of PLD is limited. Thus

the hydrolysis reaction rate will be also reduced. Correspondingly, the whole surface pressure has slow increase, indicating that PLD is more active at lower surface pressure[11].

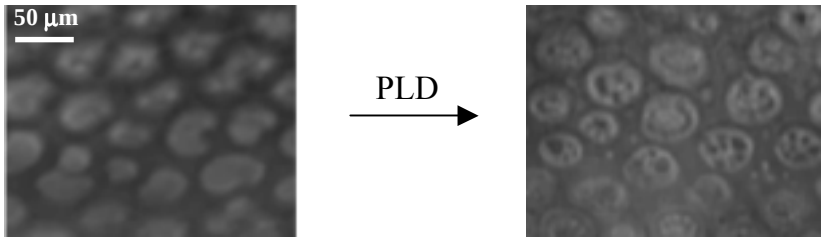
Figure 2 π-t Isotherms during the hydrolysis process of PLD to L-DPPC monolayer at different initial pressures.



(a) 4.8 mN/m; (b) 7.8 mN/m; (c) 12.5 mN/m

BAM images of pure L-DPPC monolayer at the air/water interface before and after the reaction were captured and shown in **Figure 3**. An obvious fact is that the hydrolysis reaction results in the morphology change of the lipid monolayer. In the liquid-condensed phase (LC), a circular solid domain (bright area) has been cleaved by PLD into a "lotus" structure. From BAM principle, we learned that the molecular aggregations with the same light refractive properties of LC region have the identical molecular orientations. Therefore, we can conclude that after the hydrolysis those "lotus" domains are the mixtures of DPPA produced by enzymatic hydrolysis and the nonreacted L-DPPC.

Figure 3 BAM images of domain structure change of an L-DPPC monolayer before the injection of PLD into the subphase and after the hydrolysis reaction (C_{PLD} = 0.125 U/mL, π_0 = 4.8 mN/m)



LC domains of L-DPPC domains after hydrolysis reaction

In experimental process, we found the L-DPPC domain would not immediately change into "O" or "S" types of domains as the phospholipase A_2 (PLA_2) was added into the subphase [9]. This indicates that the hydrolysis reaction of PLD to lipid monolayer is not along the same molecular orientation direction. Oppositely, it cleaves the lipid molecules at any position, and the hydrolysis products either partly dissolve into the subphase or rearrange at the interface. Meanwhile, one of the hydrolysis products, DPPA, may inhibit the reaction of cleavage to lipid monolayer and finally results in the L-DPPC domain to keep the same size and the hydrolysis reaction takes place inside the liquid condensed phase. Such a product inhibitory phenomenon was also reported in the hydrolysis reaction of PLA_2 or phospholipase C (PLC) by other groups [5, 12]. The current experimental finding is helpful in building up a theoretical model for the hydrolysis reaction of PLD to lipid and understanding the hydrolysis mechanism.

In conclusion, the hydrolysis process of the L-DPPC monolayer at the air/water interface by PLD can be directly monitored with BAM and film balance technique. An obvious "lotus" shape of domain has been observed during the hydrolysis of PLD. The analysis of the morphological changes of L-DPPC domain may provide a direct evidence of the hydrolytic mechanism of lipid monolayer. One of the cleavage products, DPPA, probably plays an important role in the regulation of the catalytic process and in the formation of new types of domain after L-DPPC cleavage.

Acknowledgments

This research was supported by NNSFC (NO.29925307) as well as the research contract between the German Max-Planck-Society and the Chinese Academy of Sciences.

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Received 4 November, 2002