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**Studies of crab digestive phospholipase acting on
phospholipid monolayers: Activation by temperature**

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Abbreviation

CDPL, crab digestive phospholipase; PPPL, porcine pancreatic phospholipase ; sPLA₂, secreted phospholipase A₂ ; PC, phosphatidylcholine; BPB, *p*-bromo-phenacyl bromide.

Abstract

The water-soluble lipolytic enzymes act at the interface of insoluble lipid substrates, where the catalytic step is coupled with various interfacial phenomena as enzyme penetration, solubilization of reaction products, loss of mechanical stability of organized assemblies of phospholipids molecule, etc. Using the classical emulsified system and the monomolecular film technique, we compared the interfacial properties of crab digestive phospholipase (CDPL) with those of the porcine pancreatic one (PPPL). A kinetic study on the surface pressure dependency of the two phospholipases was performed using monomolecular films of three substrates: di C₁₂-PC (1,2-dilauroyl-sn-glycerol-3-phosphocholine); di C₁₂-PG (1,2-dilauroyl-sn-glycerol phosphoglycerol) and di C₁₂-PE (1,2-dilauroyl-sn-glycerol phosphoethanolamine). The use of a substrate in monofilm state allows the monitoring, during the biocatalysed reactions, of several physico-chemical parameters and permits the modification of the "quality of interface". The effect of the temperature on the hydrolysis rate of these substrates was also checked. Our results show that specific activities of both phospholipases were affected by the variation of the subphase temperature. CDPL was irreversibly inactivated by p-bromo-phenacyl bromide, the specific inhibitor of secretory PLA₂s. The hyperbolic behaviour observed was coherent with hopping mode of action, one of the two characteristic interfacial mechanisms of PLA₂s.

Key words: Crab phospholipase; Phospholipid monolayer; Hydrolysis; Temperature effect; Surface pressure.

Introduction

The phospholipases [1–3] are water-soluble enzymes that can catalyze the hydrolysis of ester bonds of phospholipids. The enzymatic lipolytic reaction is an important case of heterogeneous catalysis. The water-soluble lipolytic enzymes act at the interface of insoluble lipid substrates, where the catalytical step is coupled with various interfacial phenomena as enzyme penetration, solubilization of reaction products, loss of mechanical stability of organized assemblies of phospholipids molecule, etc. [3]. The natural substrates of lipolytic enzyme are long-chain lipids generating water insoluble lipolytic products. There are at least four reasons for using lipid monolayers as substrates for lipolytic enzymes: (i) The monolayer technique is highly sensitive, and very little lipid is needed to obtain kinetic measurements. This advantage can often be decisive in the case of synthetic or rare lipids. (ii) During the course of the reaction, it is possible to monitor one of several physicochemical parameters characteristic of the monolayer film: surface pressure, potential, radioactivity, etc. These variables often give unique information. (iii) With this technique the lipid packing of a monomolecular film of substrate is maintained constant during the course of hydrolysis, and it is therefore possible to obtain accurate, presteady-state kinetics measurements with minimal perturbation caused by increasing amounts of reaction products. (iiii) Probably most importantly, it is possible with lipid monolayers to vary and modulate the “interfacial quality”, which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge densities, the water structure, the viscosity, etc. One further advantage of the monolayer technique as compared to bulk methods is that, with the former, it is possible to transfer the film from one aqueous subphase to another. The mechanism of enzyme lipolysis at the lipid/water interface is an important topic of intensive research in biochemistry. Various kinetic models have been developed to describe the mechanism of enzyme lipolysis at the lipid/water interface [1-8]. Maximum

phospholipase catalytic activity is usually expressed in the presence of a lipid/water interface. As is well known, phospholipase catalytic activity is drastically influenced by the physico-chemical properties of the interface and activation at the interface is one of the most important characteristics of lipolytic enzymes [3]. In our laboratory, crab digestive phospholipase (CDPL) was purified from crab hepatopancreas [9]. The monolayer technique was employed, in the present work, to study the influence of surface pressure and temperature on the hydrolysis rate of three phospholipids substrates by CDPL at different compression degrees. To allow comparison, porcine pancreatic phospholipase A₂ (PPPL) was studied as pancreatic phospholipase model.

2. Materials and methods

2.1. Lipids

Phosphatidylcholine (PC); di C₁₂-PC (1,2-dilauroyl-sn-glycerol-3-phosphocholine); di C₁₂-PG (1,2-dilauroyl-sn-glycerol-3-phosphoglycerol); di C₁₂-PE (1,2-dilauroyl-sn-glycerol-3-phosphoethanolamine) were from Sigma Chemical (St. Louis, USA). Phosphatidylcholine was a generous gift from Dr F. Carrière (Laboratoire d'enzymologie interfaciale et de physiologie de la lipolyse (LEIPL), CNRS, Marseille, France). All phospholipids were speared from a chloroform solution using an Agla microliter syringe unit (Burroughs-Wellcome).

2.2. Phospholipid monolayer

Phospholipid monolayers are spontaneously formed when an organic solution of phospholipids is speared on water surface [10-13]. The hydrophilic headgroups of the phospholipids face towards the water and the hydrophobic alkyl chains face towards the air. The tendency of the phospholipid molecules to self assembly forms a two-dimensional phospholipid phase. The phase state of this monolayer depends on the phospholipids concentrations at interfaces (molecules/area). If a movable barrier is used to compress the phospholipids monolayer (i.e. the decrease of the area available to the phospholipid), a two-dimensional phase diagram can be measured at constant temperature. Surface pressure (δ) as a function of the molecular area (A) (i.e. δ - A isotherm) can provide the whole information with the phase transitions [14,15]. The packing of the phospholipids molecules in a monolayer as a function of the surface pressure is shown in Fig. 1. At the beginning, the monolayer is in the sate of the gas phase (G) and the molecular arrangement is completely disorder. When the monolayer is compressed by a lateral surface force, the molecules become closer and subsequently undergo a phase change from G to the liquid-expanded (LE) phase, the coexistence region of LE and the liquid-condensed (LC) phases. Under further compression, some monolayer will also enter a solid (S) state, where the molecules are closely packed and

orderly oriented along a certain direction. With a further compress, the monolayer will be collapsed. It is noted that the shape of the isotherms depends greatly on the structure of the phospholipids. Obviously, δ -A isotherms allow statements to be made about their orientation, mobility and interactions with compounds dissolved in the subphase. There are eventually two experimental ways to investigate the interactions between lipid monolayer and enzymes at the air/ water interface. One is so-called injection method, which allows the enzyme to be injected in a subphase of a spread lipid monolayer at a constant pressure. Another is to spread a lipid monolayer at a subphase containing enzyme buffer solution. In most cases, injection method is chosen. As soon as the lipid monolayer gets a contact with the enzyme from the subphase, the hydrolytic reaction catalyzed by phospholipases will occur. The hydrolytic rate is very slow at a lower surface pressure. However, few hydrolytic products will lead to the molecular rearrangement of lipid membrane and cause much effect on the whole hydrolytic reaction according to the results obtained by spectroscopic techniques. Therefore, injection method is a better choice to the present researches.

2.3. Water

The water used was first deionized and then distilled from alkaline permanganate in an all-glass distillation apparatus. Absolute surface tension values and the absence of surface tension changes upon compression indicated that no surface-active impurities were present after cleaning the surface several times with a Pasteur pipette connected to a vacuum pump. Unless otherwise stated, all assays were performed with the following standard buffer: 10 mM Tris-acetate, pH 8.0, 100 mM NaCl, 21 mM CaCl₂ and 1 mM EDTA.

2.4. Enzymes

Crab digestive phospholipase (CDPL), was purified to homogeneity at our laboratory as previously described [9]. Pure porcine pancreatic phospholipase (PPPL) was from Sigma Chemical (St. Louis, USA).

2.5. Determination of phospholipase activity

The PLA₂ activity was measured titrimetrically at pH 8.0 and at 50°C with a pH-stat, under the optimum conditions, using purified egg-PC or a crude egg yolk emulsions as substrate in the presence of 5 mM NaTDC and 10 mM CaCl₂. Some assays were performed with NaDC. One unit of phospholipase activity was defined as 1 μmole of fatty acid liberated under standard conditions.

2.6. Monolayer technique

Before each use, the Teflon trough and barriers were cleaned first with tap water in order to eliminate all salt components of the previous experiment, and then gently brushed with distilled ethanol, in order to eliminate the enzyme or protein which can bind to the Teflon, subsequently washed again with tap water and finally rinsed with distilled filtered water. After this washing procedure, water must not wet the Teflon surface. The aqueous subphase was composed of 10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 21mM CaCl₂, and 1mM EDTA. Water used for buffer preparation was distilled and filtered on a Super Q Millipore System containing four columns: a 0.22 μm filter, an active charcoal column and an Organex column. After dissolving the salts and adjusting the pH to the desired value, the buffer was filtered again through a 0.22 μm Millipore membrane in order to eliminate all dust and bacteria which might be present. Residual surface active impurities were removed before each assay by sweeping and suction of the surface.

★ Enzyme Kinetic experiments

Measurements were performed with KSV-2000 Baro-Stat equipment (KSV-Helsinki). The principle of the method was described previously by Verger and de Haas [16]. It involves the use of a “zero-order” trough with two compartments: a reaction compartment and a reservoir compartment, which were connected to each other by a small surface channel. The enzyme solution was injected into the subphase of the reaction compartment only when the

lipid film covered both compartments. A mobile barrier, automatically driven by the Baro-Stat, moved back and forth over the reservoir to keep the surface pressure (π) constant, thus compensating for the substrate molecules that were removed from the film by enzyme hydrolysis. The surface pressure was measured on the reservoir compartment with a Wilhelmy plate (perimeter 3.94 cm) attached to an electromicrobalance, which was connected in turn to a microprocessor programmed to regulate the mobile-barrier movement. As showed previously [16], the sensitivity of the Wilhelmy plate was estimated to 0.2 mN m^{-1} (Fig. 2). The reaction compartment was stirred at 250 rpm using 2 cm magnetic stirrers. The reactions were performed at 10, 25 or 50 °C. The reaction compartment has a surface of 120 cm^2 and a volume of 120 ml. The reservoir compartment was 147 mm wide and 249 mm long. All the activity measurements were performed after 15 min of enzyme injection. Maximal activities were expressed as the number of moles of substrate hydrolyzed by unit time and unit surface of the reaction compartment of the “zero-order” trough for an arbitrary phospholipase concentration of 1M.

2.7. Determination of protein concentration

Protein concentration was determined as described by Bradford et al. [17] using BSA ($E_{1\text{cm}}^{1\%} = 6.7$) as reference.

2.8. Inhibition with *p*-bromo-phenacyl bromide

This modification was carried out essentially as described by Roberts et al. [18]. In brief, *p*-bromophenacyl bromide (BPB) was added to the sample to give a final concentration of $1 \times 10^{-4} \text{ M}$, and the mixture was incubated at room temperature for 24 h.

Results and Discussion

1. Specific biochemical properties of pure CDPL and PPPL using pH- stat method

CDPL and PPPL have millimolar calcium requirement and showed maximum activity by addition of 10 mM CaCl₂ in the reaction medium. The optimal pH and temperature were 8 and 50°C, respectively, for both CDPL and PPPL. In contrast to PPPL, CDPL was completely inactivated at 60°C. The specific activity of CDPL reaches 40 U/mg using phosphatidylcholine (PC) as substrate in the presence of 5 mM NaTDC and 10 mM CaCl₂ at pH 8 and 50°C. Using the same substrate, the specific activity of PPPL is higher than this of CDPL, and reaches 1000 U/mg in the presence of 10 mM CaCl₂ and 4 mM NaTDC at pH 8 and 50°C. Moreover, CDPL hydrolyses the PC less efficiently than PPPL since the ratio representing the catalytic efficiency (K_{cat}/K_m) is ten times higher with PPPL than with CDPL (data not shown).

2. CDPL acts in the *hopping mode*

The activity as a function of the substrate concentration shows an hyperbolic curve indicating that the activity level was sensitive to the bulk substrate concentration (data not shown). Consequently, CDPL acts in the hopping or jumping mode [19]. In this mode, crab digestive phospholipase is able to hydrolyse all the phospholipids present at the aggregate developing a highly efficient hydrolytic process. Otherwise, in a scooting mode of action, no Michaelian behaviour could be observed, because at catalytic concentrations, CDPL remains adsorbed during the catalytic cycle and could only hydrolyzes the outer phospholipid monolayer. Further increase in phospholipid concentration has no effect on the enzyme activity in the scooting mode [19].

2. Enzyme kinetics in monolayer studies

2.1. Variations with surface pressure of CDPL and PPPL activities using phospholipids monolayers

It is well established that phospholipases A_2 from venom are able to hydrolyse phospholipids monolayer films even at high surface pressure (30-40 $\text{mN}\cdot\text{m}^{-1}$) and this allow them to attack biological membranes [20]. In order to check the ability of crab digestive phospholipase to penetrate and to hydrolyze phospholipids monolayer films, we reported the rate of hydrolysis of three phospholipids substrates as a function of the surface pressure for the CDPL (Fig. 3). To allow comparison, we reported, in the same figure, the results obtained with the porcine pancreatic phospholipase. As can see from Fig. 3A, both CDPL and PPPL present a maximum activity with bell-shaped surface pressure curves when di C_{12} -PC was used as substrate. Fig. 3A also shows that CDPL hydrolyzes di C_{12} -PC monolayer less efficiently than PPPL does. A maximal ratio of about 1.6 was found between catalytic activities of the two phospholipases, measured at their surface pressure optima (10 and 15 $\text{mN}\cdot\text{m}^{-1}$, respectively) at 25°C. At the same temperature (25°C), when using di C_{12} -PG and di C_{12} -PE monolayers as substrates, the CDPL and PPPL activities increase continuously with surface pressure then reaches a plateau at about 15 $\text{mN}\cdot\text{m}^{-1}$ and 20 $\text{mN}\cdot\text{m}^{-1}$ for di C_{12} -PG and di C_{12} -PE, respectively (Fig. 3B, 3C). An increase in monolayer surface pressure caused an increase in the number of molecules per unit of area and an increase in the number of reactive collisions, which may explain the influence of surface pressure on the hydrolysis rate. Qualitative interpretations have been given to explain this phenomenon. The first hypothesis was that a packing-dependent orientation of the substrate may regulate lipolytic reactions [21]. Another interpretation explained the surface pressure maximum in terms of changes in phospholipase conformation during interface adsorption: an optimal conformation can be achieved at intermediate values of monolayer surface pressure [22]. In fact, low and high

values of surface pressure could lead to phospholipase inactivation or to the prevention of enzyme adsorption at the interface [23]. One can say that the initial surface pressure changes will cause the phase variations of the monolayer by both the molecular rearrangement and phospholipase adsorption. Moreover, the electrostatic interactions at interface are involved in the interaction between lipids and enzymes as the buffer role [24]. Based on the classification established by Verheij et al [20], CDPL and PPPL belong to group III of PLA₂ which includes some microbial and pancreatic phospholipases A₂.

2.2. Influence of temperature on the catalytic activities of CDPL and PPPL using phospholipids monolayers

As is well known, the monomolecular film technique was used to study the dependence of lipolytic enzymes on the surface pressure. But to our knowledge no information about the influence of temperature on lipolytic enzymes activity is available using the monomolecular film technique. We plotted in Fig. 3 the rate of hydrolysis of phospholipid films as a function of the temperature in the case of crab digestive and porcine pancreatic phospholipases. As can be seen from this figure, the CDPL and PPPL activities increased with temperature independently of the substrates and the surface pressures used. In fact, no change on the surface pressure optima of the two enzymes can be observed (Fig. 3A). The increased activity with rising temperature is more significant in the case of PPPL when di C₁₂-PC monolayer was used as substrate. As it was observed at 25°C, CDPL hydrolyzes di C₁₂-PC monolayer less efficiently than PPPL at high temperature. A maximal ratio of about 4 was found between catalytic activities of CDPL and PPPL at 50°C.. When using di C₁₂-PG and di C₁₂-PE as substrates, CDPL and PPPL activities increase with surface pressure to reaches a plateau at about 15 mN.m⁻¹ and 20 mN.m⁻¹ for di C₁₂-PG and di C₁₂-PE, respectively at 50°C (Fig. 3B and 3C). The same behaviour was obtained at 25°C. The maximal ratios of about 3.5 and 3 were found between catalytic activities for CDPL and PPPL, respectively, when

increasing the temperature from 25°C to 50°C using di C₁₂-PG monolayer as substrate. However, at the same conditions and when di C₁₂-PE was used as substrate, these maximal ratios were found to be about 2.2 and 3.8 between catalytic activities for CDPL and PPPL, respectively (Fig. 3B, 3C). It is worth noticing, that no catalytic activity was detected at 10°C on all these substrates (data not shown). When temperature caused acceleration of enzyme catalyzed reaction rate is generally consistent with the Arrhenius or Eyring theory [25–29]. The Eyring model takes into account formation of a transition state enzyme–substrate complex at the free energy maximum along the reaction pathway. The reactant molecules must overcome this energy barrier. In general the lower the energy barrier is the faster the overall reaction will proceed. If reaction proceeds through two or more steps, the one that has the highest free energy will often be the rate determining step.

2.3. Inhibition of CDPL and PPPL with *p*-bromo-phenacyl bromide using pH- stat method

Bromo-phenacyl bromide (BPB) is a specific inhibitor of phospholipases that covalently links to histidine groups at the active site [18]. Our results show a complete inhibition when 100 µl of BPB 100 mM was added to 0.5 ml of active CDPL or PPPL fractions, while the addition of 20 µl only showed partial inhibition (Fig. 4). The reaction was pH dependent. At pH below 6, BPB didn't affect the activity of the enzyme (data not shown). This means that a histidine group, with a pK of 6.1, is involved. The inactivation was developed by keeping the enzyme solution in a buffer at pH 8 during 6 h.

Conclusion

We summarize that lipid monolayer is a good model to study the membrane hydrolysis mechanism catalyzed by phospholipases at interface. The results show that the hydrolysis yield and rate depend on different initial surface pressures or phase state of lipid monolayer. The maximum activity of phospholipases locates in the LE/LC coexistence region. Generally, at higher lateral pressures, the phospholipid molecules are tightly packed and the penetration on the active site of the enzyme is difficult, whereas at low pressures, the enzyme penetrates more easily into the lipid monolayer. Such threshold pressure is observed for the two phospholipases. The threshold pressure depends on the structure of the enzyme, the nature of its active site, the electrostatic conditions and physiochemical state of phospholipids. In all experiments, a lag phase is observed after the addition of the enzyme. The so-called lag-burst behaviour may be a general phenomenon for hydrolysis reactions catalyzed by phospholipases. Thus, the recognition and cleavage are considered as two basic steps for the hydrolysis reaction. The present study shows also that temperature influences the activity of CDPL and PPPL on phospholipids monolayers. Enzyme activity increases by increasing temperatures up to 60°C, but above this value, thermal inactivation occurs.

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Legends to figures:

Fig. 1. Pressure/area diagram of a lipid monolayer: representation of the compression behaviour and packing in the gas (G), liquid-expanded (LE), liquid-compressed (LC), and solid (S) phases.

Fig. 2. Schema of the “zero-order” Teflon trough.

Fig. 3. Variation with surface pressure in CDPL and PPPL activities measured on di C₁₂-PC (A), di C₁₂-PG (B) and di C₁₂-PE (C) at two different temperatures (25°C) and (50°C). Specific activity is expressed as the number of moles of substrates hydrolyzed by unit time and unit surface of the reaction compartment of the “zero-order” for an arbitrary lipase concentration of 1M in the aqueous subphase of this compartment. Activity measurements were performed after 15 min of PLA₂ injection under di C₁₂-PC monomolecular films. The kinetic experiments were recorded as described in materials and methods section. Final enzyme concentration in the reaction compartment was 0.2 nM. Buffer: 10 mM Tris-HCl, pH 8.2, 150 mM NaCl, 21 mM CaCl₂, and 1mM EDTA.

Fig. 4. Effect of the concentration of *p*-bromo-phenacyl bromide (BPB) on CDPL and PPPL activities using pH-stat method. Enzyme activities were measured at various concentrations of BPB using PC emulsion as substrate at pH 8 and at 50°C in the presence of 5 mM NaTDC and 10 mM CaCl₂ for CDPL and 4 mM NaTDC and 10 mM CaCl₂ for PPPL.

Fig. 1.

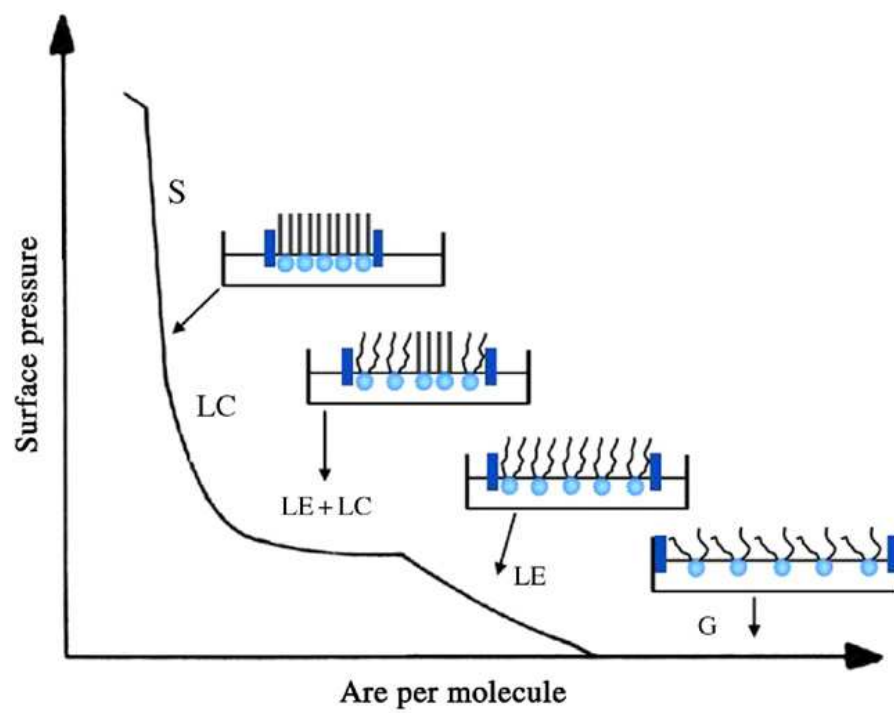


Fig. 2.

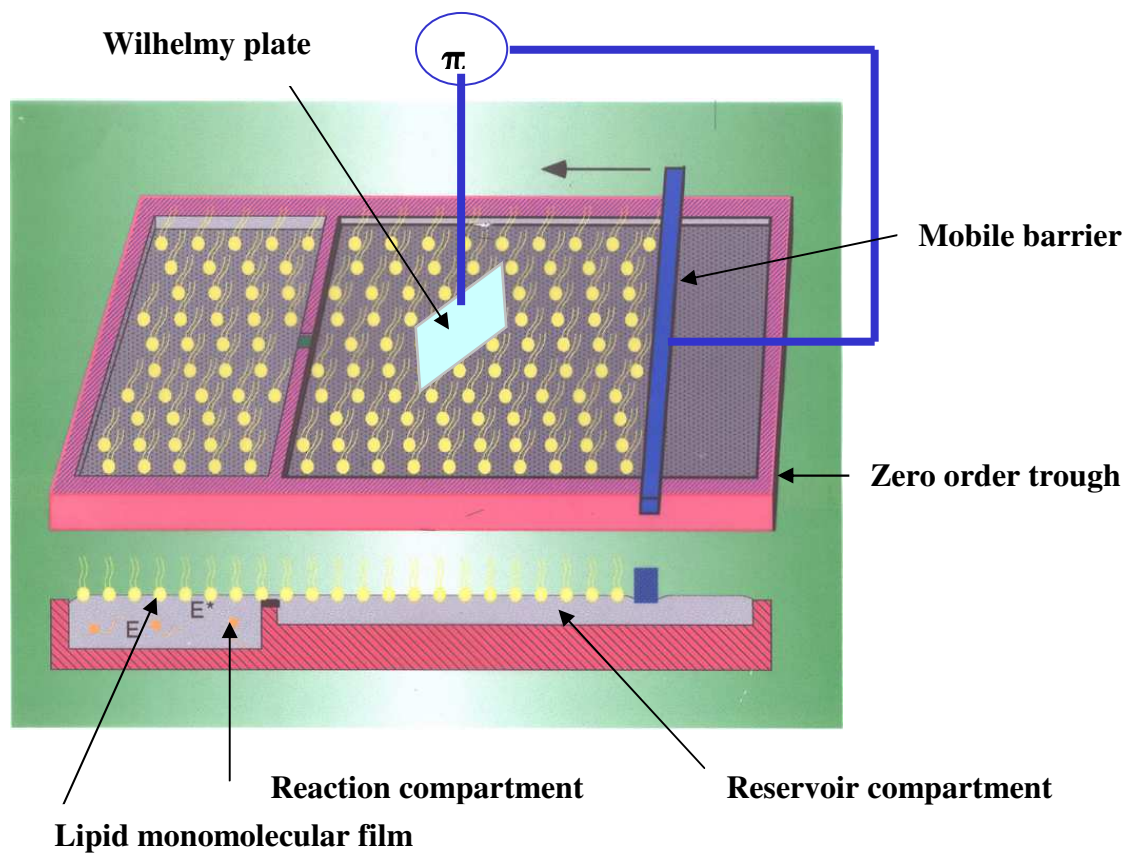
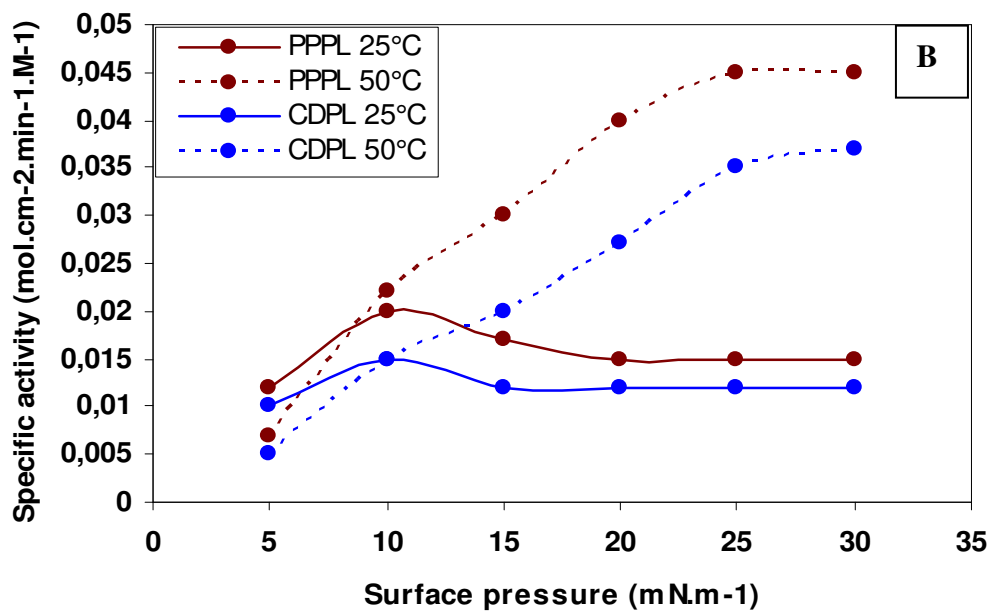
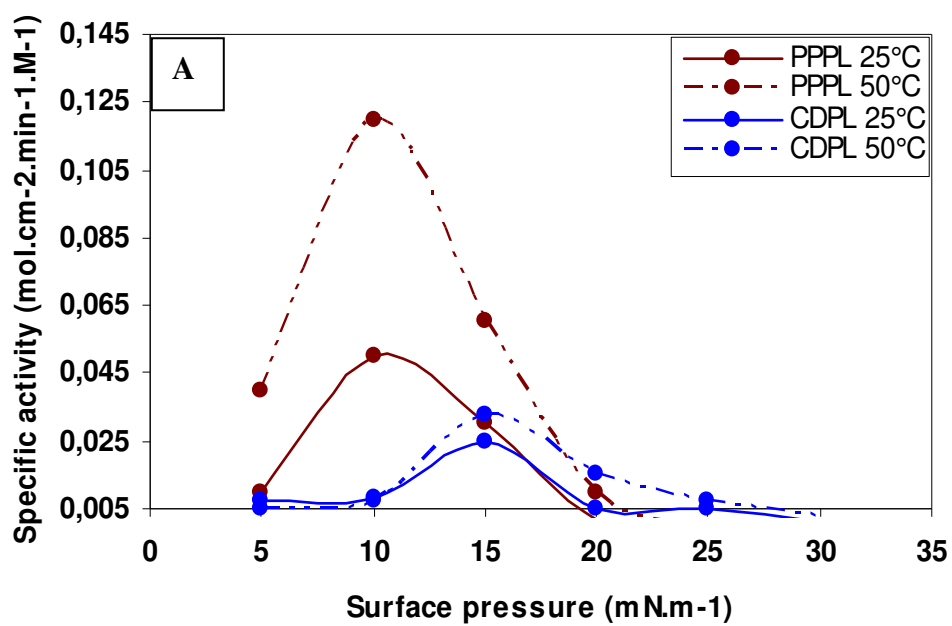


Fig. 3.



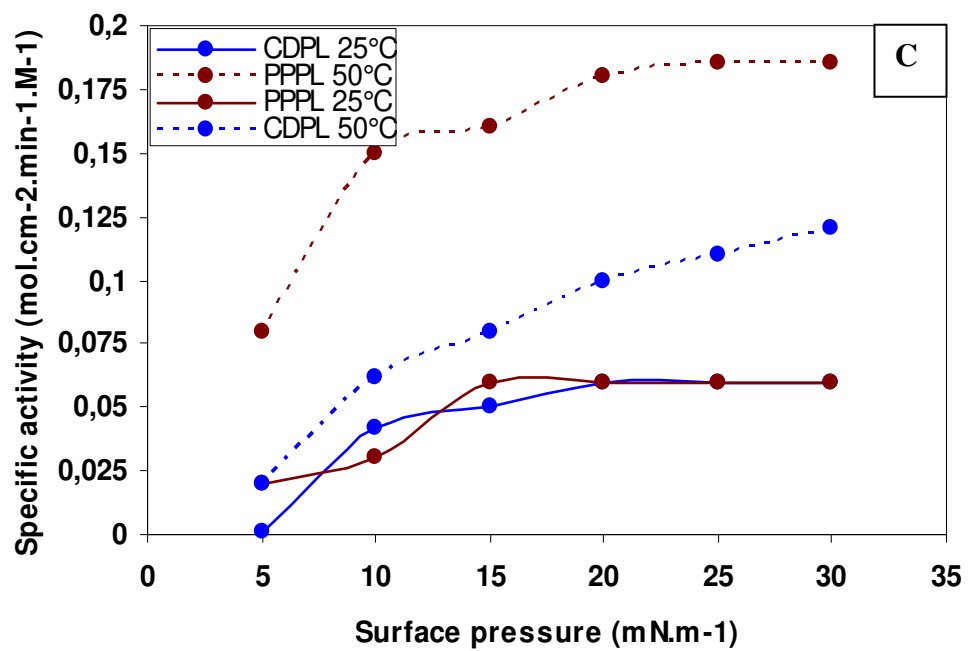


Fig. 4.

