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Phospholipids hydrolysis in organic solvents catalysed by immobilised phospholipase C

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Abstract

Phospholipase C from *Bacillus cereus* has been immobilised on XAD7, Sepabeads FP-DA, Eupergit C, Celite 547, Silica gel 60 by covalent attachment or by adsorption. Preparates obtained with the two different immobilisation techniques show good hydrolytic activity in water-saturated organic solvents with phosphatidylcholine (PC) as a substrate. The chiral 1,2-diacyl glycerol can easily be obtained from the organic phase. The catalysts obtained by covalent attachment have been prepared with a higher specific activity and are suitable for repeated uses, while the ones prepared by adsorption with lower specific activity can only be used once. The initial rates of hydrolysis of PC solutions in different organic solvents and water content are compared.

Keywords: Phospholipase C; Enzyme immobilisation; Diacylglycerols; Organic solvents

1. Introduction

Phospholipases C (PLC) are hydrolytic enzymes catalysing the hydrolysis of natural phospholipids to diacylglycerol (DAG) and organic phosphate (Fig. 1). These enzymes can be isolated from micro-organisms of different genus, namely from *Clostridium* [1] and *Bacillus* [2–5]. *Bacillus cereus* produces several different PLC with different specificity. The enzymes able to hydrolyse phosphatidyl inositol (PLC_{PI}) [6] and phosphatidyl choline (PLC_{PC}) [7] have been isolated and characterised. These enzymes can be advantageously exploited in biocatalysis to obtain valuable compounds like DAG [10] and organic phosphates by simple hydrolysis. Indeed hydrolysis of phospholipids, natural or unnatural with different polar head groups gives access to a number of organic phosphates which are difficult to obtain by other methods [8].

Applications of these enzymes in synthetic or mechanistic experiments employ PLC in an organic/water biphasic system. In these conditions the enzyme is not recycled and a difficult separation of the two phases often hampers the

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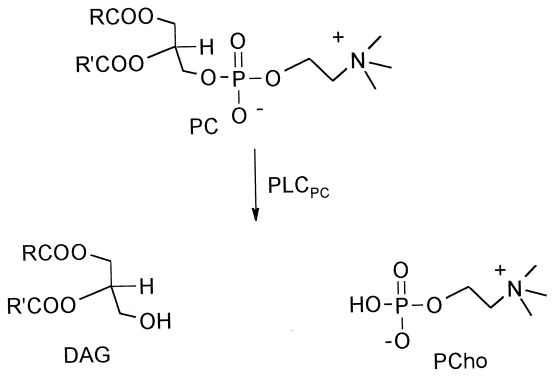

Fig. 1. Products in the PLC catalysed hydrolysis of PC.

practical exploitation of the procedure. The lipase catalysed hydrolysis of triglicerides has been investigated for the preparation of DAG, useful as additive in the food industry. In this case mixtures of racemic 1,2-diglycerides and 1,3-diglycerides are obtained. Since stereoselectivity strongly depends on the nature of the acyl chains, only in the case of non-natural triglycerides with three identical acyl chains, lipase stereoselectivity is tuneable to give enantiomerically enriched 1,2(2,3-)-DAG [9]. The PLC catalysed hydrolysis of PLs gives instead 1,2-diglycerides with the enantiomeric composition of the natural product if acyl migration can be prevented. [1] It has recently been shown that enantiomerically pure 1,2-diglycerides can be used for the preparation of phospholipid analogues with retention of the natural configuration and without acyl migration [10]. We have studied the application of PLC immobilised on a solid support for the hydrolysis of phospholipids in an organic solvent at controlled water content. With this technology there should be advantages in the production of DAG:

- the enzyme can be utilised in a more efficient way,
- the virtual absence of water as a solvent should control acyl migration [11],
- continuous production should in principle be possible,
- recovery of product easier than in an emulsion system.

In contrast a water-organic solvent biphasic system appears more suited for the obtainment of the water soluble part of the hydrolysis mixture i.e., the organic phosphate (phosphorylcholine, PCho, when PC is used as a substrate, like in this case).

Moreover the preparation of immobilised PLC is of interest for further applications in the study of the mechanism of this enzyme.

- Substrate specificity independent from the aggregation state. Since phospholipases catalyse the hydrolysis of water insoluble substrates, it has been shown or sometimes assumed that they act at a biphasic interface and that hydrolysis rate depends on the aggregation state [5,12–14]. Hydrolysis experiments catalysed by an immobilised enzyme in the presence of water in nearly stoichiometric amount will give a rank of substrate specificity which is independent from possible interfacial activation or activity. [2]
- Transesterification ability of PLC$_{PC}$. Unlike the case of the biotransformation of phospholipids catalysed by other phospholipases [15,16], the transesterification capacity of PLC has not been observed in an emulsion system. The use of immobilised PLC preparates active in organic solvent should allow the study of the possible transesterification activity by suppressing the otherwise prevalent hydrolysis reaction.

2. Materials and methods

2.1. Materials

PC from soy beans of 95% purity (Epicuron 200) was obtained from Lucas-Meyer (FRG). PLC was obtained from the culture broth of an

[1] PLC hydrolysis of phospholipids has previously been used for the determination of the absolute configuration of the glycerol backbone. We assume that the enantiomeric composition of the DAG obtained in this work is the same found in natural PC.

[2] Work in progress from this laboratory.

overproducing strain of *B. cereus*. The bacterium was grown in a Chemap fermentor in a 7.5 l vessel on a conventional medium [3,17]. The enzyme is released extracellularly and the specific activity of the solution can be brought to 10 U ml^{-1} by an enrichment method based on collecting the foam generated by passing a gas stream into the enzyme solution [17] (one unit is defined as the amount of enzyme hydrolysing 1 μmol of PC from egg-yolk per min). The preparate could be used as such or further purified as described in the literature to give an enzyme solution of 1000 U ml^{-1}.

Amberlite XAD7 was supplied from Rohm and Haas. Sepabeads FP-DA was obtained from Mitsubishi. Eupergit C was from Rohm Pharma. Celite 547 was from Fluka. Silica gel 60 was from Merck.

1,2-diaminoethane and glutaraldehyde solution were from Fluka. All solvents used were reagent grade distilled prior to use.

2.2. Methods

Enzymatic activity was measured as the hydrolysis rate of an emulsion of PC from egg yolk in a pH-stat at pH 7 as described [2,3].

2.3. PLC immobilisation by adsorption

2.3.1. Preparates R and Q

PLC adsorption on celite and silica gel. The material (500 mg) was suspended in 5 ml of culture broth enriched by foaming to 10 U ml^{-1}, at pH 7.5. The suspension was gently stirred occasionally and left overnight at room temperature. The mixture was then dried under vacuum at room temperature.

2.4. Covalent immobilisation of PLC on solid support

2.4.1. Preparate L

Five hundred milligrams of *sepabeads*, an amino residue containing resin, was treated with a 10% solution of glutaraldehyde in water for 12 h. The solid was thoroughly washed with water and mixed with a solution of 0.5 mg of purified PLC (1200 U mg^{-1}) dissolved in 3 ml of 0.05 mM borate buffer at pH 7.5 and 0.1 mM in Zn^{2+}. The suspension was kept at 25°C for 2 h. The solid was filtered on a sintered glass funnel. The solution contained about 50% of the original activity. The solid was resuspended in 2 ml of borate buffer and treated in portions with excess $NaBH_4$ at 2°C during 30 min. After filtration the polymer was washed with water until neutral, dried on paper and then under high vacuum. The preparate was used as described in a following section.

2.4.2. Preparate O

XAD-7 was treated at reflux with ethylendiamine for 4 h and then filtered and washed with *n*-hexane [18]. The dried resin was then treated with the glutaraldehyde solution as described for the preparate L and used in a similar way.

2.4.3. Preparate T

Eupergit C, an oxirane activated resin, was directly treated with 3 ml of a 0.1 M borate buffer solution containing 0.5 mg of PLC (1200 U mg^{-1}) for two days. The mixture was occasionally stirred and then filtered and stored wet at 2°C.

2.5. PC hydrolysis for initial rate determination

Fifty milligrams of PC were dissolved in 1 ml of water-saturated toluene and 25 mg of preparate L were added. The mixture was shaken on a vibrator at 200 rpm at 25°C.

The progress of the PC hydrolysis was monitored by measuring the GC amount of DAG formed with *n*-heptadecane as an internal standard with the following procedure: 0.1 ml sample was withdrawn from the reaction mixture and 0.1 ml of a standard solution of *n*-heptadecane in toluene (20 mg ml^{-1}) was added. The sample was diluted with toluene to a total vol-

ume of 1 ml and 1 μl of this solution was filtered through celite and injected. GC apparatus HP 6890 with automatic controller G1512A and G1513A injector. Ten meter capillary glass column coated with SE52 (MEGA, Italy) 0.32 mm i.d., film thickness 0.1/0.15 μm. Temperature programme was 90°C 1 min, 20°C min^{-1} 250°C 2 min, 15°C min^{-1} 300°C 2 min, 20°C min^{-1} 350°C. Detector FID at 300°C. Injector at 300°C. Carrier He, 1 bar. DAG was detected as an equilibrated signal of 1,2- and 1,3-diglycerides at retention time 15.2 and 16.3 min. Internal standard *n*-heptadecane, rt 5.25 min.

For further uses preparates O and L were filtered, resuspended in borate buffer filtered and dried in vacuum. The other preparates were filtered and dried in vacuum.

2.6. PC hydrolysis on a preparative scale

Two grams of preparate L were suspended in 80 ml of a 70 mM water-saturated toluene solution of PC and shaken at 25°C. The reaction was complete after 60 min as monitored by TLC. The mixture was filtered on a sintered glass funnel and the organic phase eluted through a short silica gel column from which after solvent removal 2.8 g of DAG (> 90%) was obtained.

2.7. Experiments at defined water content

Preparates L and Q were suspended in water saturated toluene and stored for 4 h at 25°C in a close daisycutter in the presence of water. The solid was filtered in a sintered glass funnel and resuspended in 1 ml of 70 mM PC solution in water saturated toluene to which additional water in the amount described in Fig. 4 was added.

3. Results and discussion

Two different kind of immobilisation techniques were used:

- adsorption.
- covalent immobilisation.

For use in organic solvents, adsorption methods are usually employed. The production of DAG in our case is accompanied by the formation of organic phosphates (i.e., choline phosphate, PCho) not soluble in organic solvents. It is expected that this compound will remain adsorbed on the catalyst with possible modification of the catalytic properties. It is therefore advisable to wash with water or buffer the catalyst after each cycle in order to remove the phosphate. In this case, enzymes covalently immobilised should retain the catalytic activity which could otherwise be washed away in the case of adsorbed catalysts.

Adsorbed catalysts were prepared employing directly the culture broth obtained by simple removal of the biomass by centrifugation. The specific activity of the preparates (R and Q) is therefore limited from the yield of the enzyme in the fermentation. The same enzyme source was also used in the preparation of T in which eupergit C was used as a carrier. In the preparation of the other two immobilised catalysts O and L, the enzyme was linked covalently to the carrier. In these cases a highly purified and highly active enzyme was used. The two groups of catalysts had similar catalytic properties except for the possibility of recycling as shown in Fig. 2. As expected covalently linked PLC can be used for several cycles. Table 1 collects data

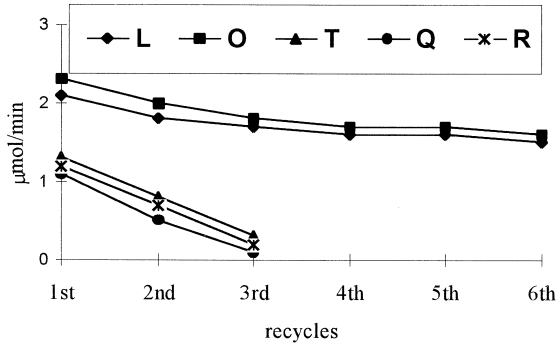

Fig. 2. Initial rates of PC hydrolysis in water-saturated toluene (1 ml, 70 mM) for the various PLC immobilised preparates (25 mg).

Table 1

Preparate	Solid support	Loaded enzyme ($U\ g^{-1}$)[a]	Bound enzyme (%)[b]	Specific activity ($U\ g^{-1}$)[c]	Catalytic activity (%)[d]	mg PC/g catalyst[e]	Recycles[f]
Q	celite	100	25	2	8	300	1
R	silica gel	100	20	2.5	10	300	2
O	XAD7	600	68	84	10	2000	4
T	eupergit C	100	25	1.2	5	300	1
L	sepabeads	600	75	92.8	21	2000	6

[a] PLC units per dry weight of carrier.
[b] Based on the residual activity in the immobilisation solution.
[c] Displayed units per dry weight of carrier determined from the initial velocity at 15 min of PC hydrolysis in water saturated toluene (70 mM) at 25°C.
[d] Compares the activity as PLC units initially bound to the carrier and displayed in the hydrolysis reaction.
[e] Milligram of PC which can be completely converted per g of catalyst.
[f] Additional uses resulting in complete PC conversion (see Fig. 2).

concerning the amount of enzyme initially loaded and the percentage recovered in the solution. From initial velocity data we then assigned the % catalytic activity as the activity displayed in the hydrolysis reaction in comparison with the bound enzyme. It appears that catalysts obtained by adsorption (Q and R) from which a lower specific activity is expected on the basis of the loaded enzyme, show instead relatively higher catalytic activity in comparison to the preparates obtained by covalent immobilisation (O, T, L). The explanation for the observed behaviour can only be found in generic and unspecified protein-carrier interaction favouring the two inorganic materials or in protein modification occurring during the immobilisation practice and especially during the reductive step. However, the activity covalently bound proved to be much less affected by the reaction condition since with preparates O and L recycling with high efficiency has been obtained (Fig. 2). The preparates with a lower initial activity were not suitable for reuse, and this can be easily explained with the different treatment for the two group catalysts after each cycle. In fact preparates Q, R and T were filtered, dried and reused, while O and L were washed with buffer dried and reused. In this step, PCho which is adsorbed or bound to the carrier, can probably be removed. This is not possible in the 1st group type carrier in which the activity is therefore rapidly declining after the first use. The temperature effect is only marginal (Fig. 3) and shows the usual trend observed in enzymatic catalysis.

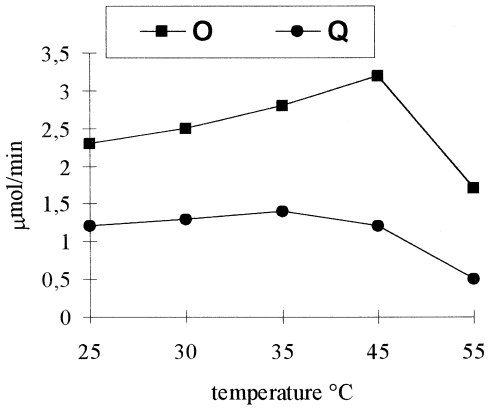

Fig. 3. Temperature effect on the initial rates of PC hydrolysis (μmol min^{-1} of product formed) in water-saturated toluene (1 ml, 70 mM) for PLC immobilised preparates types O and Q (25 mg).

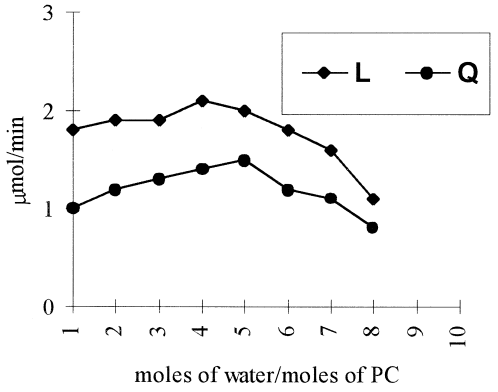

Fig. 4. Effect of water on initial rates of PC hydrolysis in water-saturated toluene (1 ml, 70 mM) for PLC immobilised preparates types L and Q (25 mg).

XAD7 has been used before for the covalent immobilisation of hydrolytic enzymes [18]. Sepabeads is an industrial resin for protein separation which has probably been used for the first time in this work for this purpose. The effect of water activity [19] was not investigated in detail. When the preparate was thoroughly dried, obviously, no activity could be detected. The activity observed after hydration measured as initial rate of PC hydrolysis was slightly increased when the amount of added water to the suspension was raised from 1 to 4 H_2O/PC moles ratio. Further increase of the ratio influenced negatively the reaction rate (Fig. 4) regardless of the solvent used. The fact that activity was observed after hydration is significant since it allows the belief that the preparate retains enzymatic activity when dried and it can be used in experiments of transesterification in the absence of stoichiometric water with an organic nucleophile. [3] The effect of different

[3] Work in progress from this laboratory.

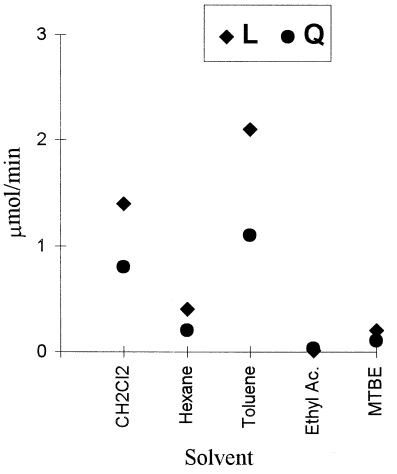

Fig. 5. Initial rates of PC hydrolysis (μmol min^{-1} of product formed) in water-saturated organic solvents (1 ml, 70 mM) for PLC immobilised preparates L and Q.

water-saturated organic solvents on the initial rate of hydrolysis was investigated (Fig. 5). In all the experiments toluene proved to be the solvent of choice as far as initial reaction rates are concerned. Moreover in other solvents like in hexane, the reaction becomes very slow after 40–50% conversion.

4. Conclusion

PLC_{PC} immobilised on solid phases has been prepared for the first time and applied to the hydrolysis of PC in organic solvents with a slightly more than the stoichiometric amount of water. Products of PC hydrolysis are DAG which is recovered from the organic solution, and PCho which is retained on the solid phase. With preparates O, T and L where the enzyme is covalently linked, the organic phosphate can be washed desorbed with water and the preparate reused several times. In the case of the enzymatic preparations Q and R where the enzyme is merely adsorbed, water treatment is not possible and the immobilised enzyme rapidly loses its activity. The obtainment of the phosphate part is better achieved with a biphasic system. The catalytic activity of the immobilised PLC_{PC} in organic solvents in the presence of nearly stoichiometic water should allow experiments for the study of the hitherto undetected transesterification capacity of this enzyme, in analogy with PLC_{PI} [15].

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