PHOSPHOLIPIDS IMMOBILIZED ON BEADED AGAROSE BY HYDROPHOBIC INTERACTION AS HYDROPHILIC SUBSTRATES FOR PHOSPHOLIPASE C

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1. Introduction

Many different assay procedure for phospholipases have been used [1,2] e.g., with egg yolk [3], red blood cells [4] and thromboplastin [5] as nonspecific substrates and liposomes [6,7], micelles [8] and monolayers of phospholipids [9] as more specific substrates. The nonspecific methods are difficult to use when mixed enzyme preparations are analysed due to the possibility of unwanted interactions between different substrates and enzymes. The results from assays based on purified phospholipids, with or without detergents, are difficult to reproduce since they are dependent on micelle charge and size [8,10–12]. Monolayer techniques have been used for a long time [13] but are technically demanding [14].

Herein an immobilized substrate for phospholipase C is described. The phospholipids are adsorbed on the amphiphilic gel octyl–Sepharose CL-4B [15], which is easy to handle in water systems. The phospholipids are probably sparsely adsorbed on the octyl groups in the gel with their hydrophilic parts outwards as in membranes, which makes them accessible to phospholipases C in a standardized way.

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2. Materials and methods

2.1. *Immobilization of phospholipids*

2.1.1. Transfer of agarose gels to organic solvents

For adsorption of sphingomyelin, octyl–Sepharose CL-4B (lot no. 9080) and Sepharose CL-4B (lot no. 0666, Pharmacia Fine Chemicals, Uppsala) were carefully washed in distilled water with increasing concentrations of isopropanol (p.a. grade), (25%, 50% and 75% (v/v)) on a glass filter funnel [15]. For adsorption of phosphatidylcholine, the gels were first transferred to acetone by successive washings in 33%, 66% and 100% (v/v) acetone (purum grade). Thereafter, they were transferred in the same way to chloroform–methanol, 2:1, by successive washing in 33%, 66% and 100% (v/v) chloroform–methanol, 2:1, (p.a. grade) in acetone.

2.1.2. Adsorption of sphingomyelin

To *n* g gel (wet wt) in 2 *n* ml 75% isopropanol was added 5 *n* mg sphingomyelin (bovine brain type 1, Sigma, St Louis, MO), dissolved in a minimal amount of pure isopropanol. The gels were kept in suspension by a slow end-over-end rotation of the vessel. The suspensions were then diluted with distilled water stepwise and allowed to equilibrate for 15 min after every dilution step. The isopropanol concentrations in the steps were 75%, 54%, 43%, 35%, 26% and 17%. After dilution to 17% isopropanol, the suspension was equilibrated before being washed successively with water, 2 M NaCl in 0.1 M acetate buffer, pH 4.5, 2 M NaCl in 0.1 M Tris–HCl buffer, pH 8.5, and finally water. Thereafter the gel was suspended in 2.5 *n* ml water.

2.1.3. Adsorption of phosphatidylcholine

To *n* g gel (wet wt) in chloroform–methanol 2:1 was added 5 *n* mg phosphatidylcholine (dipalmitoyl, grade 1, synthetic, Sigma) dissolved in 1.6 *n* ml of the same solvent. The gels were kept in suspension by end-over-end rotation and then diluted stepwise with acetone. The equilibrium time after every step was 1 h and the chloroform–methanol (2:1) concentrations were 100%, 66%, 33%, 17% and 8% (v/v in acetone). After the last step the gels were transferred to water by washings in 100%, 66%, and 33% acetone (v/v in water). The gels were further washed as described for gel with adsorbed sphingomyelin in section 2.1.2 and finally suspended in 2 *n* ml H_2O.

2.2. *Determination of adsorbed phospholipids*

Water-suspended phospholipid-gels (0.1–0.4 ml) were dried by heating and 0.4 ml perchloric acid (p.a. 70%) was added. The gel was boiled in an acid-washed test tube until colourless. A further 0.2 ml of perchloric acid was added and the amount of phosphorus was measured [17].

2.3. *Phospholipase C assay*

Phospholipase C from *Clostridium perfringens* was purified as in [3] and had a specific activity of 340 IU/mg protein as measured in a pH-stat with egg yolk as substrate [3].

Phospholipid-gel was suspended in 20 mM Tris–HCl buffer, pH 7.4, containing 10 mM $CaCl_2$ and 1 mM $ZnCl_2$. The phospholipase C was diluted in the same buffer and 10–30 μl was added. The final incubation volume was 1 ml for sphingomyelin-gel and 2 ml for phosphatidylcholine-gel. The assays were performed in a water bath at 37°C under vigorous shaking to keep the gels in even suspensions. After enzyme digestion the gel was quickly filtered (Polypropylene Econo-Columns, Bio-Rad Labs., CA) and 2 units of alkaline phosphatase (Calf intestinal mucosa, 990 U/ml, Sigma) were added to the water-phase [16]. This enzymatic splitting of phosphorylcholine proceeded for 75 min at 37°C. Enzymatic hydrolysis of standard phosphorylcholine (calcium salt, Sigma) controls was included with each series of assays. The amount of liberated inorganic phosphorus was then determined [17]. The amount of water-soluble phosphorus released in this test system from the phospholipid-gel without enzyme and the phosphorus content of the enzyme were subtracted from the test results.

All chemicals were obtained from Merck A. G., Darmstadt, if not otherwise stated and all preparative steps were performed at room temperature.

3. Results

Table 1 shows that the hydrophobic octyl–

Table 1
Amounts of phospholipids adsorbed to agarose gels

Type of phospholipid	Type of agarose	Phosphorus adsorbed (μmol/g dry gel)
Sphingomyelin	octyl–Sepharose CL-4B	102
	Sepharose CL-4B	18
Phosphatidylcholine	octyl–Sepharose CL-4B	47
	Sepharose CL-4B	7

Table 2
Accessibility of adsorbed phosphatidylcholine to hydrolysis by phospholipase C

Assay phase	Released phosphorus after max. hydrolysis by phospholipase C	
	(μmol P/g dry gel)	(%)
octyl–Sepharose CL-4B		
gel phase	37	79
water phase	9.5	21
Sepharose CL-4B		
gel phase	6.0	81
water phase	1.4	19

Sepharose CL-4B adsorbed 5–7 times more phospholipids than the ordinary Sepharose CL-4B gel. About 60% of added sphingomyelin and 25% of added phosphatidylcholine were adsorbed to the hydrophobic gel under the conditions described.

In order to compare the accessibility of the adsorbed phosphatidylcholine as substrate to phospholipase C in the 2 different gels, samples of these were incubated for 3 h with 2.2 μg phospholipase C. Under these conditions, maximal phospholipid hydrolysis was attained in this assay. As shown in table 2, about 20% of the adsorbed phosphatidylcholine was hydrolyzed independent of gel type.

The effect of enzyme concentration and the time courses of enzymatic hydrolysis of phosphatidylcholine and of sphingomyelin are demonstrated in fig.1 and fig.2, respectively. After initial rapid rises the rates of hydrolysis of the phospholipid-gel substrates appeared to be linear both with regard to enzyme concentration and to time.

4. Discussion

Phospholipids are amphiphilic molecules which form liposomes in water systems [18]. The size and shape vary markedly with the experimental conditions and these variations influence the reproducibility when they are used as enzyme substrates. By adsorbing monomers of phospholipids to a solid hydrophobic surface a uniform distribution of molecules can be expected.

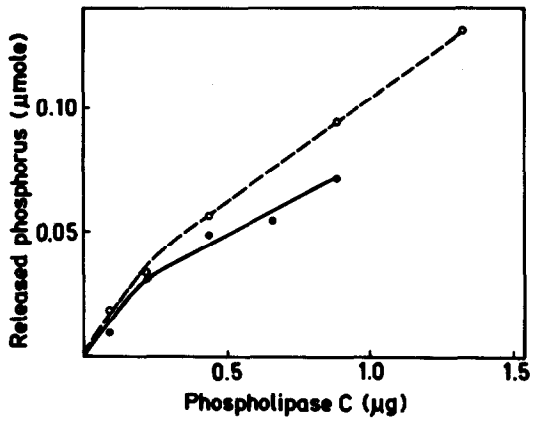

Fig.1. Octyl–Sepharose gel with 1.15 μmol adsorbed phosphatidylcholine or 1.06 μmol adsorbed sphingomyelin was incubated with different amounts of phospholipase C. Incubation times were 20 min for sphingomyelin (o- - - -o) and 30 min for phosphatidylcholine (•- - - -•), both at 37°C.

The advantages of agarose gels as a solid support are the high porosity, the large surface area and the hydrophilic character of the gel network. The introduction of octyl groups on the gel by the glycidyl ether method [15] yields a noncharged hydrophobic derivative, which is important in order to minimize electrostatic interactions upon adsorption of phospholipids. Agarose gels can be transferred to most

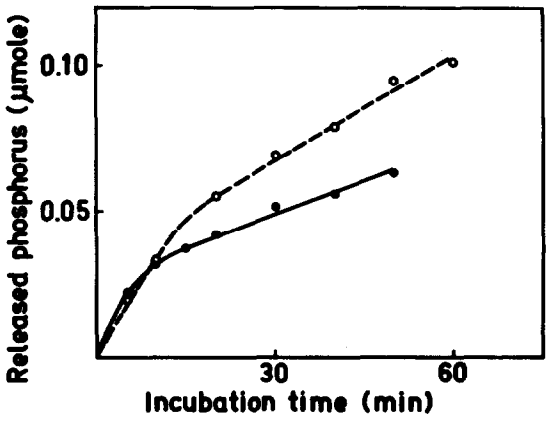

Fig.2. Phospholipase C (0.44 μg) was incubated for different times with sphingomyelin–octyl–Sepharose (o- - - -o) and phosphatidylcholine–octyl–Sepharose (•- - - -•) as in fig.1.

organic solvents by successive washing in a series of solvents of decreasing polarity. By choosing suitable solvents for the different hydrophobic phospholipids it is possible to adsorb them onto the hydrophobic (octyl) groups. This adsorption is due to partition between the gel and the solvent which is slowly increased in polarity. This is important for uniform distribution and high adsorption of the phospholipids to the gel beads. Some adsorption takes place in unsubstituted agarose gel but it can probably be decreased by improving the conditions for the adsorption and washing steps.

Due to the large pores in Sepharose CL-4B, most enzymes with mol. wt $< 10^5$ would be expected to be able to penetrate the gel beads. Nevertheless, only about 20% of the adsorbed phosphatidylcholine on both types of gels was hydrolysed by phospholipase C under these conditions (table 2). This fact may indicate that large parts of the substrate were adsorbed on the gels at positions where it could not be reached by the enzyme. The results also indicate a similar distribution of substrate in the two types of gel.

One may envision that the outer parts of the gel beads are first penetrated by the enzyme and that the hydrolysis product from this part of the substrate has a shorter diffusion distance than the product formed in the inner part of the gel. This is probably the reason for the apparent faster reaction in the beginning of the experiments shown in fig.1 and fig.2. After this, a state is reached where the substrate is hydrolysed linearly with time and enzyme concentration. Another explanation for the 2 phase rate of the hydrolysis might be that the ζ potential of the substrate changes as hydrolysis proceeds [19].

The system is very reproducible and can be useful for characterization and comparison of purified enzymes. Due to the hydrophilic character of the gel, it is easy to suspend in water systems and the separation of product from substrate is performed by a simple filtration. Crosslinked agarose is very stable [20] and the temperature, buffer and different salts can be varied over wide ranges. The sensitivity of the described system cannot be better than that of the phosphorus assay, but by using radioactively labelled phospholipids it should be possible to increase both the speed and the sensitivity of the analysis.

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