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Purification of phospholipase C by hydrophobic interaction affinity chromatography[a]

E. C. S. CHAN, I. R. SIBOO and R. SIBOO*

Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, Quebec H3A 2B4 (Canada)

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ABSTRACT

A simple procedure is described for the purification of phosphatidylcholine-hydrolyzing phospholipase C(PLC). Lecithin, the substrate for PLC, was ligated hydrophobically to octyl-Sepharose in 2 *M* $(NH_4)_2SO_4$. The washed lecithin-conjugated resin was then used to purify PLC from crude preparations by affinity chromatography. PLC binds to the lecithin moiety in the presence of Zn^{2+} and is eluted with an acidic buffer containing EDTA. PLC activity was recovered in the eluate. Both sodium dodecyl sulphate polyacrylamide gel electrophoresis and p*I* electrofocusing showed that the eluate contained a single monomeric protein with an apparent molecular mass of 66 kDa and a p*I* of 5.5.

INTRODUCTION

The phosphatidylcholine-hydrolyzing phospholipase C (PLC; phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3) is produced by oral spirochetes [1] which are putative pathogens of periodontitis [2–4]. Since PLC was detected in gingival crevicular fluid [1], it might contribute to the progression of periodontal lesions. However, the microbiota of diseased periodontal pockets consist not only of oral spirochetes but also other bacterial species as well as leukocytes that migrated into diseased pockets. Consequently, the PLC in crevicular fluid might have originated from leukocytes and/or bacteria other than spirochetes. This necessitated the purification of PLC, from an oral spirochete, *Treponema denticola* serovar C, and to prepare an antiserum to the PLC of serovar C to identify spirochete-derived PLC in crevicular fluid. Various methods have been used previously to obtain pure PLC. These included affinity chromatography in which egg-yolk proteins were conjugated to cyanogen bromide (CNBr)-activated Sepharose 4B [5], ion-exchange chromatography [6] and preparative isoelectric focusing [7]. It was pointed out [6], however, that neither of these methods "has yielded an electrophoretically pure enzyme". We tried to purify spirochetal PLC

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by affinity chromatography with CNBr-activated Sepharose 4B conjugated with egg-yolk proteins but this method did not produce pure PLC. Therefore we were obliged to develop an alternative method to purify PLC.

In this report, L-α-phosphatidylcholine (α-lecithin), the specific substrate for PLC, was ligated hydrophobically to octyl-Sepharose to form an affinity resin on which PLC could be purified from crude PLC preparations. For simplicity, the technique will be referred to as hydrophobic interaction affinity chromatography (HIAC).

EXPERIMENTAL

Materials

Octyl-Sepharose CL-4B, *p*-nitrophenylphosphorylcholine, pI markers (pH 3.55–9.3), L-α-phosphatidylcholine (α-lecithin), phenylmethylsulphonyl fluoride and N-tosyl-L-phenylalanine chloromethyl ketone were purchased from Sigma (St. Louis, MO, USA); acrylamide, bisacrylamide, tetramethylethylenediamine (TEMED), ammonium persulphate, riboflavine 5′-phosphate, ampholytes (pH 3.4–9.2), silver staining kit and protein assay kit were purchased from Bio-Rad Labs. (Mississauga, Canada) and Sephadex G25 from Pharmacia (Dorval, Canada).

Growth of Treponema denticola serovar C

T. denticola serovar C was isolated originally in this laboratory [8] and deposited in the American Type Culture Collection and carries the accession number ATCC 35404. Serovar C was grown under anaerobic conditions in "new oral spirochete" (NOS) medium as described previously [1,8]; the growth medium was freed of spirochetes by cold (4°C) centrifugation (20 min, 15 000 *g*), and Na_2-EDTA (0.01 *M*) added to the supernatant. Solid ammonium sulphate was added to the supernatant to precipitate the proteins at 70% saturation (w/v). The proteins were solubilized in 0.9% NaCl, pH 7.5 (adjusted with 0.01 *M* NaOH) and the protease inhibitors, N-tosyl-L-phenylalanine chloromethyl ketone (0.5 m*M*) and phenylmethylsulphonyl fluoride (1 m*M*) were added. The protein solution was desalted with Sephadex G25 by a batch technique, and the proteins were recovered after filtration through a sintered-glass funnel; protease inhibitors were added again, and the solution dialyzed against 0.01 *M* Tris-HCl, pH 7.2 containing 2 m*M* $ZnCl_2$ at 4°C for 4 h.

Protein determination and PLC assay

Protein concentrations were determined with the Bio-Rad protein assay kit by a microassay in microtitration plates and the dye shift read at 600 nm. The PLC assay [9] was carried out in microtitration plates [1] and the *p*-nitrophenol released from the hydrolysis of *p*-nitrophenylphosphorylcholine was read at 405 nm. The absorbance of both assays was measured with a Bio-Rad Model 2550 EIA reader (Bio-Rad).

Hydrophobic ligation of α-lecithin to octyl-Sepharose

A known volume of octyl-Sepharose suspension was filtration-washed on a sintered-glass funnel with 50 volumes of glass-distilled water and then with 10 volumes of 2 *M* $(NH_4)_2SO_4$. Suction was applied to remove the aqueous mobile phase and the wetted gel was resuspended, to twice its original volume, in 2 *M* $(NH_4)_2SO_4$ solution. Lecithin (10 mg) was solubilized in 1.5 ml of a chloroform–methanol (2:1, v/v) solution. A suspension of the octyl-Sepharose was stirred gently and the solubilized lecithin added dropwise (10 mg lecithin per ml of resin). This amount of lecithin (previously determined) is in excess of the amount required to saturate (occupy) the octyl groups of the octyl-Sepharose in a 1.0-ml suspension of the gel. The mixture was stirred for an additional 30 min at 37°C (to enhance hydrophobic ligation). Free lecithin was removed by reduced-pressure filtration in a sintered-glass funnel; the resin was washed with 10 volumes of 2 *M* $(NH_4)_2 SO_4$ and repeatedly with 0.5 *M* NaCl containing 1 m*M* Zn^{2+} until the filtrate was free of lecithin. To determine that the α-lecithin remained ligated to the resin, the presence of lecithin in the filtrate was determined by extracting 1 volume of the filtrate with 1 volume of chloroform (twice) and the amount of lecithin in the pooled extracts determined from a standard curve (see below). The aqueous phase of lecithin-conjugated resin was removed (as above) to obtain the wetted resin, and several aliquots (1 g) were taken. Two samples were each extracted with 2 ml of chloroform (twice), and the amount of lecithin in the pooled chloroform extracts of each sample was determined from a standard curve.

Standard curve

Lecithin was solubilized in chloroform (20 μg/ml to 1 mg/ml) and the absorbance measured at 375 nm (DuModel spectrophotometer, Beckman Instruments, Fullerton, CA, USA).

Affinity purification of PLC with lecithin-octyl-Sepharose

Several 1-g samples of wetted gel were used in a batch-type technique to determine whether or not the starting and elution buffers would dissociate the lecithin from the resin and what effect Zn^{2+}, Ca^{2+}, EDTA, salt concentration and pH had on binding and elution of PLC to and from the affinity resin. The optimal conditions, once established, were used to purify PLC from the crude enzyme preparation on a 2.0 cm × 1.0 cm I.D. column. The lecithin-conjugated resin was packed into the column and washed with 0.01 *M* Tris–HCl buffer containing 0.5 *M* NaCl and 1 m*M* Zn^{2+}. The crude PLC preparation (60 U/mg of protein) was allowed to percolate into the resin and left in contact with the resin at room temperature for 5 min before the starting buffer was applied at a flow-rate of 3.0 ml/min. Samples of 20 ml were collected until the absorbance of the eluent returned to baseline and the bound protein eluted with an elution buffer (0.2 *M* glycine, pH 3.0 buffer containing 0.5 *M* NaCl and 1.5 m*M* Na_2EDTA). Samples of 3.0 ml were collected in tubes with 120 μl of 1 *M* Tris and the absorbances

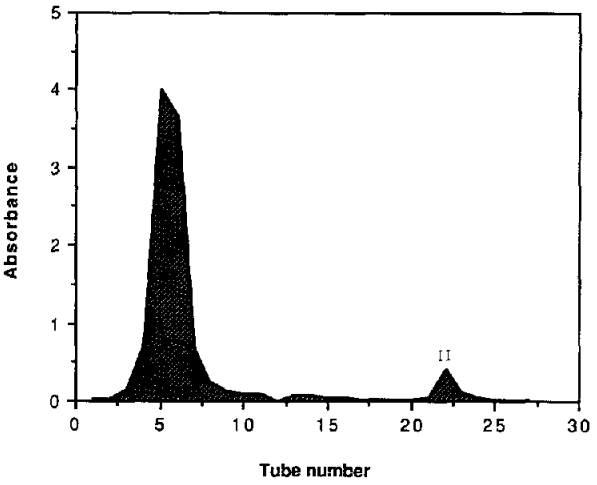

Fig. 1. Hydrophobic interaction affinity chromatography. A crude preparation of PLC was applied to the α-lecithin-conjugated resin, and the bound PLC eluted with a buffer (0.2 *M* glycine, pH 3.0 containing 0.5 *M* NaCl and 1.5 m*M* EDTA). PLC activity was recovered in peak II (right). The elution profile is based on absorbance at 280 nm.

recorded at 280 nm. The eluent of peak II (Fig. 1) were pooled, desalted and concentrated by reduced pressure dialysis (protein concentration unit, Fisher Scientific, Dorval, Canada) and assayed for PLC activity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous electrophoresis technique [10] was used with the Mini-Protean II cell (Bio-Rad). The separating gel buffer (1.5 *M* Tris–HCl, pH 8.8), the stacking gel buffer (0.5 *M* Tris–HCl, pH 6.8), the acrylamide-bis (30% T, 2.67% C) solution and sample buffer, which contained only 3% β-mercaptoethanol, were filtered (0.22 μm, Millipore, Bedford, MA, USA) just before use to reduce streaking and non-specific lines in the stained gel [11,12]. The sample (15 μg) was separated under reducing and non-reducing conditions, with a 4% stacking–10% separating gel at 200 V until the bromophenol dye reached the distal end of the gel. The gels were fixed and silver-stained with the Bio-Rad stain kit.

Isoelectric focusing

Horizontal electrofocusing was carried out in 5% polyacrylamide gels with a Mini IEF cell (Bio-Rad). The focusing gels were prepared with the following reagents: 2.0 ml of acrylamide monomer solution [24.25% (w/v) acrylamide, 0.75% (w/v) bisacrylamide], 5.5 ml of double-distilled water, 2.0 ml of 25% (w/v) glycerol and 0.5 ml of 40% (w/v) ampholine. Polymerization was initiated with 15 μl of 10% (w/v) ammonium persulfate, 50 μl of 0.1% (w/v) riboflavin 5′-phosphate and 3 μl of TEMED and the polymerization of the cast gel completed by

ultraviolet light activation of the catalysts. The sample (1 μg) was applied at the anodic and cathodic sides of the gel and focused with a programmable-step-mode (100 V for 15 min, 200 V for 15 min and 450 V for 60 min) Model 1000/500 power supply (Bio-Rad). The gels were fixed and stained in a solution of 0.04% Coomassie blue R-250, 0.5% $CuSO_4$ and 0.5% Crocein scarlet in 27% isopropanol.

RESULTS

Ligation of lecithin to octyl-Sepharose

Ligation of lecithin to the resin, in 2 *M* $(NH_4)_2SO_4$, was recognizable by the yellowish-brown color of the resin after it was washed extensively. The amount of lecithin bound to octyl-Sepharose was quantified by extraction of the lecithin from the lecithin-conjugated resin with chloroform. One gram of the wetted-gel binds 28 mg of lecithin. The lecithin does not dissociate from the lecithin-conjugated resin with buffers used for the purification of PLC. The limit of sensitivity for the detection of lecithin with the standard curve is ≤ 10 μg/ml.

Affinity purification of PLC

Preliminary studies of the purification of PLC by the batch technique showed that Ca^{2+}, in the starting buffer, enhanced the binding of PLC to the lecithin-conjugated resin. The bound PLC could not be eluted, in high yield, with elution buffer. The eluate contained 12% and the lecithin-conjugated resin 66% of the total PLC activity added to the resin. Attempts to elute the bound PLC with 0.5 *M* $(NH_4)_2SO_4$, 0.15 *M* NaCl and water were not fruitful. When detergents such as Triton-X 100 and Tween 20 were used dissociation of the ligand from the octyl-Sepharose was evident. When Zn^{2+} ions were used in the starting buffer, PLC activity in the eluent and on the lecithin-conjugated resin was 74 and 6%, respectively. In no instance was it possible to recover the total activity of the PLC used in these experiments. It appeared that the low pH of the elution buffer might have denatured some of the PLC protein. Others have reported that PLC from other bacterial species have an optimum pH of 6.6–8.0 [5,6,13], and a low pH such as 5.5 partially inactivates phosphatidylinositol-specific phospholipase C (PIC) during chromatography [14].

The affinity separation of the crude PLC preparation produced an eluted peak (peak II, Fig. 1) that contained 76% of the applied PLC activity. It is essential that the elution buffer contains EDTA and the size of the separating column be selected to reduce the contact time between the PLC and the acidic buffer. Inactivation of some PLC activity occurred in spite of the fact the eluent was collected in 1 *M* Tris. Run-to-run recovery activity of the PLC varied from 64 to 81%.

SDS-PAGE and pI electrofocusing

The protein(s) of the enzymatically active elution peak II contained a single monomeric protein with an apparent molecular mass of 66 kDa (Fig. 2) under

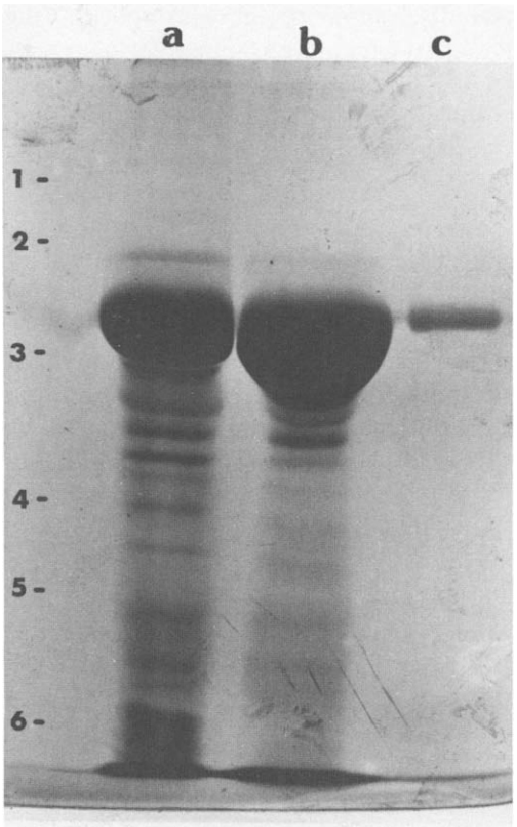

Fig. 2. SDS-PAGE of $(NH_4)_2SO_4$ precipitate of spirochete-free growth medium (lane a), spirochete-free medium concentrated by reduced-pressure dialysis (lane b) and protein of peak II (lane c). The molecular mass markers in kDa are 1 = 130, 2 = 75, 3 = 50, 4 = 39, 5 = 27 and 6 = 17. The gel was silver-stained.

reducing and non-reducing conditions. This was also apparent from the single band on electrofocusing the protein of peak II. The p*I* of this protein was 5.5 (Fig. 3). The convex shape of the focused sample may be due to the instability of the enzyme in ampholine as reported for PLC isolated from *Clostridum perfringens* [7]. It is concluded that HIAC cleanly separates PLC from the crude PLC preparation and that PLC activity in elution peak II is due to a single monomeric protein.

DISCUSSION

The specific PLC substrate (α-lecithin) can be simply ligated hydrophobically, in 2 *M* $(NH_4)_2SO_4$, to octyl-Sepharose. The ligation could be followed visually. This approach is much simpler than chemically linking lecithin to spacer molecules on an inert resin. The hydrophobic interaction between lecithin and octyl-

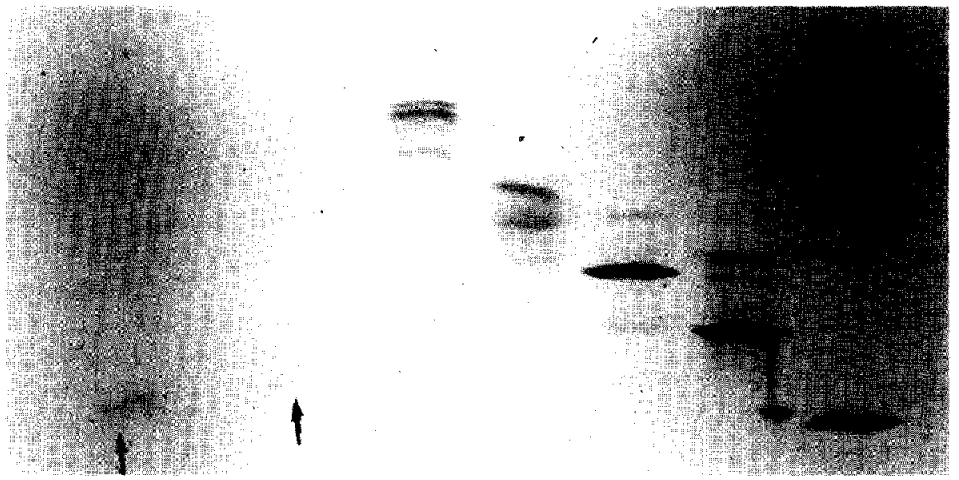

Fig. 3. Isoelectric focusing of protein form peak II. Samples were applied at the anodic and the cathodic sides of the gel. The mean p*I* was 5.5 (arrow). The other lanes contain p*I* markers .

Sepharose is maintained with 0.5 *M* NaCl in both buffers. We speculate that the lipid moiety of lecithin interacted hydrophobically with octyl groups on the octyl-Sepharose resin, and the phosphorylcholine groups interacted ionically with PLC in the presence of Zn^{2+} (Fig. 4). Ca^{2+} in the starting buffer appears to enhance the binding of PLC to the lipid moiety of lecithin. Ca^{2+} has been shown to enhance PLC binding to lecithin [15], and the elution buffer with 0.5 *M* NaCl may, in addition, actually enhance the hydrophobic interaction between PLC and the lipid moiety of lecithin. It appears that the acidic elution buffer denatures

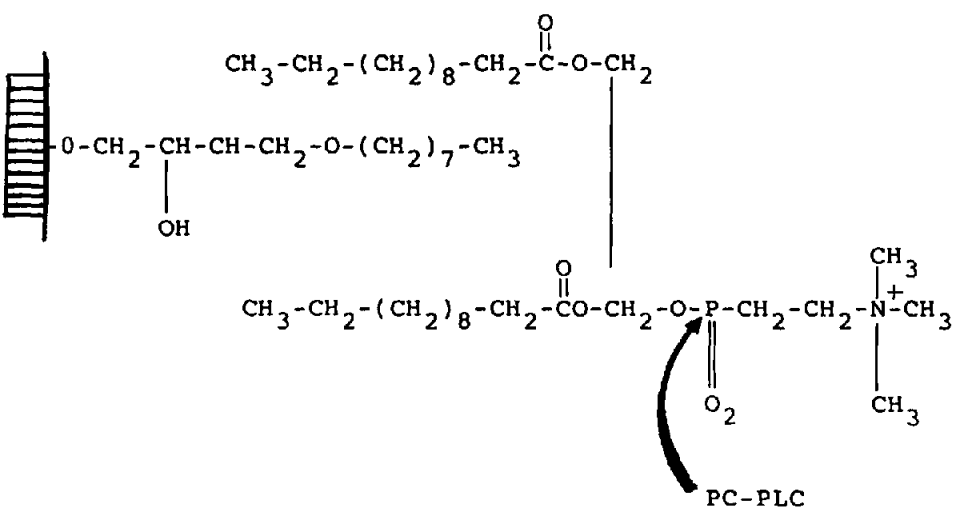

Fig. 4. Pictograph of the hydrophobic ligation of α-lecithin to the octyl group of the solid matrix (ladder-like structure); the phosphorylcholine head group is free to interact with PLC (PC-PLC).

some of the PLC protein even though small columns were used and the eluates were collected in 1 *M* Tris. Lecithin on the affinity gel was not hydrolyzed during the purification of PLC (data not shown). This is probably due to the low rate of hydrolysis of lecithin by PLC [9], the acidic elution buffer with EDTA (inhibits PLC activity), the small size of the column and the high flow-rate of the elution buffer. Although lecithin was not hydrolyzed during the chromatographic run, we did not use the same column of affinity gel for more than four chromatographic runs. It was observed, from earlier studies, that the affinity gel darkens after six or seven chromatographic runs. This darkening of the gel was due to auto-oxidation of the lecithin and this could result in its decomposition.

The apparent molecular mass of 66 kDa of the pure PLC from *T. denticola* serovar C (ATCC 35404) is similar to that reported for PLC of *Pseudomonas aeruginosa* [6] but not for PLC of other bacterial species [5,7,13]. The p*I* of 5.5 for PLC from *T. denticola* serovar C (ATCC 35404) is very similar to the p*I* of 5.2–5.7 reported for the PLC from *P. aeruginosa* [6] and *Clostridium perfringens* [5].

A simple method was used to ligate α-lecithin hydrophobically to octyl-Sepharose and to affinity-purify PLC. It is more prudent to use small affinity columns to purify PLC since the contact time with acidic elution buffers partially inactivates PLC and PIC [14]. Batches of pure enzyme have been prepared and will be used to prepare polyclonal antibody. PLC activity of crevicular fluid will be determined with and without antibody produced to PLC from *T. denticola* serovar C.

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