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Lipase and Phospholipase Protocols

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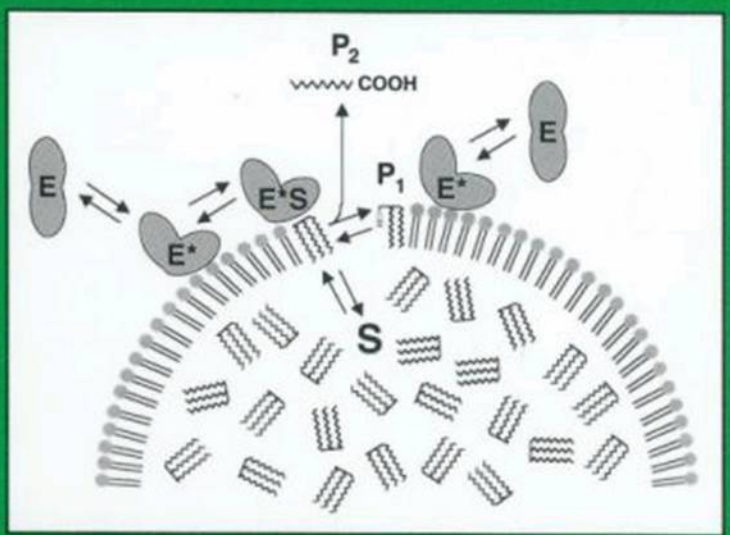

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Phospholipase A2 and Phosphatidylinositol-Specific Phospholipase C Assays by HPLC and TLC with Fluorescent Substrate

H. Stewart Hendrickson

1. Introduction

Lipolytic enzymes have traditionally been assayed by radiometric and titrimetric methods *(1)*. Radiometric methods are quite sensitive but require expensive radiolabeled substrates and tedious separation of labeled substrate and products. In addition, the safe use of radioactive materials is of increasing concern. Titrimetric assays are continuous and quite straightforward and use natural substrates but suffer from low sensitivity and are subject to conditions that may alter the amount of free hydrogen ions released Fluorescence-based assays have sensitivities that approach those of radiometric methods; although they require synthetic fluorescent-labeled substrates, they are often more convenient and rapid. For a recent review, see Hendrickson *(2)*.

We first used dansyl-labeled glycerol ether analogs of phosphatidylcholine as substrates for the assay of enzymes of the platelet-activating factor (PAF) cycle in peritoneal polymorphonuclear leukocytes *(3)*. This became a general method for the assay of phospholipase A_2 (PLA_2) (*see* **Fig. 1**; **refs.** *4* and *5*). This method can be modified to assay other enzymes of the PAF cycle such as lyso-PAF acyltransferase, lyso-PAF acetyltransferase, and PAF acetylhydrolase *(3)* Since the probe remains attached to the glycerol backbone, simultaneous assay of all of these enzymes is possible.

The method described here for the assay of PLA_2 uses thin-layer chromatography (TLC) to separate products from substrate, and quantitation by fluorescent scanning. The use of high-pressure liquid chromatography (HPLC) with fluorescence detection is included as an alternative to TLC. The assay is spe-

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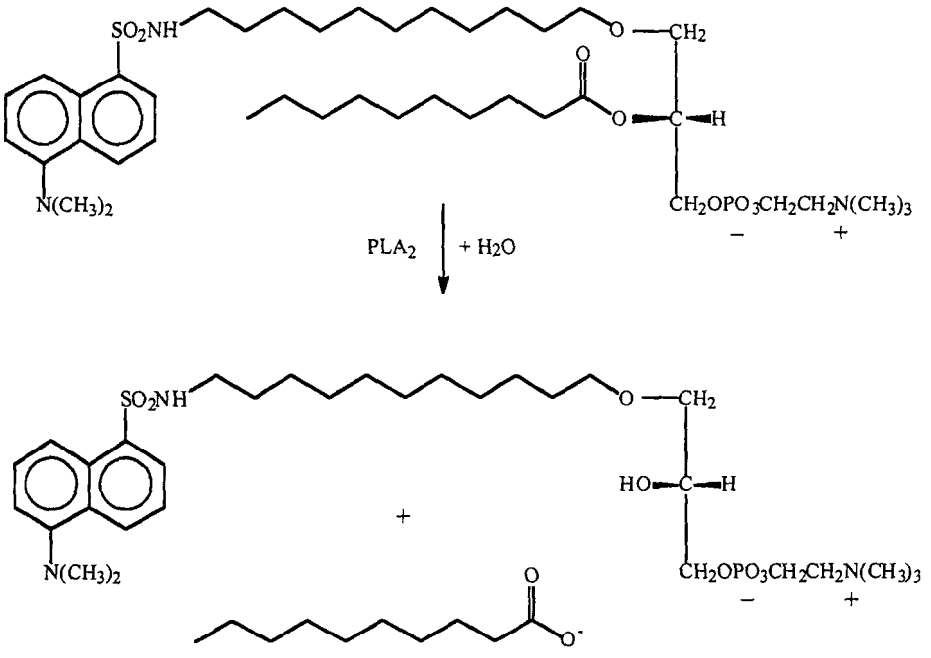

Fig 1 Reaction scheme for the assay of PLA_2 with dansyl-PC.

cific for PLA_2 since the substrate, with an ether linkage at the *sn*-1 position of glycerol, is not hydrolyzed by PLA_1. Hydrolysis of the substrate by phospholipases C or D present in crude enzyme preparations will be apparent as additional products that will be seen by TLC or HPLC

Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* catalyzes the hydrolysis of PI to a diglyceride and 1D-*myo*-inositol-1,2-(cyclic)phosphate ***(6)*** The latter is subsequently slowly hydrolyzed by the same enzyme to 1D-*myo*-inositol-1-phosphate. This enzyme also catalyzes the release of a number of enzymes linked to glycosylphosphatidylinositol membrane anchors ***(7)***

Several years ago we synthesized 4-(1-pyreno)butylphosphoryl-1-*myo*-inositol (pyrene-PI) as a substrate for the assay of PI-PLC from *B. cereus* (*see* **Fig. 2**; **refs.** ***8*** and ***9***) The method described here uses reverse-phase HPLC with fluorescence detection to separate and quantitate the product released.

The methods described here are well suited to the assay of crude enzyme preparations since the presence of other phospholipase activities will be apparent. The methods are independent of the specific conditions for the enzyme reaction, so a variety of detergents can be used. These assays can also be automated by the use of an autosampler for HPLC and larger plates with multiple

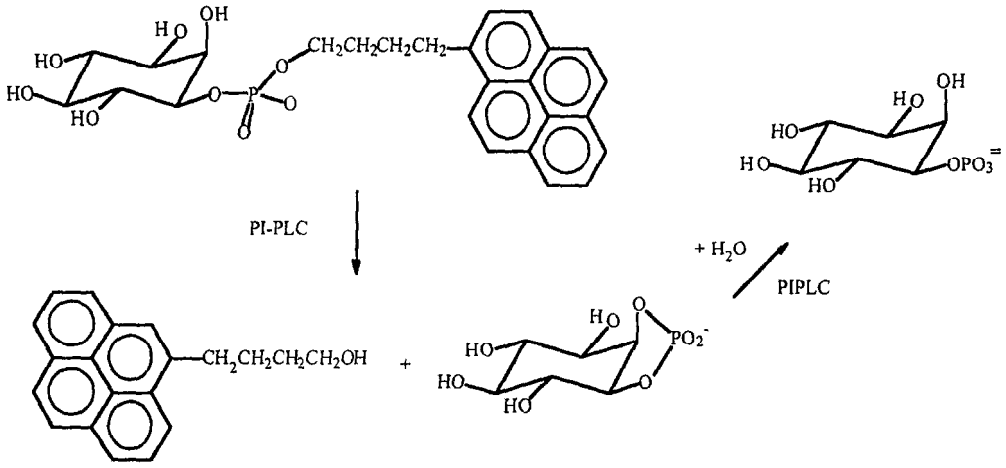

Fig 2 Reaction scheme for the assay of PI-PLC with pyrene-PI

lanes for TLC; they can thus be used to screen many enzyme samples and potential inhibitors. The TLC-based assays can done in a qualitative manner by simply visualizing the plates under an ultraviolet (UV) lamp

2. Materials

2.1. PLA_2 Assay

1 PLA_2 buffer. 0.395 *M* NaCl, 66 m*M* Tris, 13.2 m*M* $CaCl_2$, pH 7 0 (adjust with HCl)
2 PLA_2 substrate dansyl-PC, 1 m*M* in $CHCl_3$ Dissolve 1 mg of dansyl-PC (cat no D-3765, Molecular Probes, Inc , Eugene, OR) in 1 23 mL of chloroform (*see* **Note 1**) Store in a brown bottle at –20°C (*see* **Note 2**)
3 Triton X-100 solution, 10 m*M* Triton X-100 (cat no. T9284, Sigma, St. Louis, MO) in water
4 PLA_2 stock assay solution. place 100 µL of PLA_2 substrate (1 m*M* dansyl-PC) in a small test tube (10 × 75 mm) and dry under a stream of nitrogen and then under high vacuum for 10–15 min to remove all traces of solvent Add 20 µL of Triton X-100 solution (*see* **Note 3**) and 380 µL of PLA_2 buffer Vortex and sonicate (bath-type sonicator) repeatedly until the lipid is completely dissolved and the solution is optically clear.
4 TLC solvent· $CHCl_3/CH_3OH$/conc ammonia/water (90·54 5.5:2 [v/v])
5 TLC plates. 10 × 10 cm, HPTLC plates, (cat. no. 60077, Analtech, Newark, DE).
6. Fluorescence scanner: densitometer (model CS9000, Shimadzu) with fluorescence accessory, or other suitable instrument for fluorescence scanning of TLC plates.
7 Quenching solvent· hexane/isopropanol/acetic acid (6:8:1.6)
8. PLA_2 HPLC solvent hexane/isopropanol/water (6 8·1.6)

2.2. PI-PLC Assay

1. PI-PLC buffer. 50 m*M* 2-(N-morpholino)ethane sulfonic acid (MES, Sigma), pH 7 0 (adjust with NaOH)
2. PI-PLC substrate 1 m*M* pyrene-PI in $CHCl_3/CH_3OH$ (2.1). Dissolve 0 5 mg of racemic 4-(1-pyreno)butylphosphoryl-1-*myo*-inositol (cat no P-3764, Molecular Probes) in 1 25 mL of solvent Store in a brown bottle at –20°C (*see* **Note 2**)
3. PI-PLC stock assay solution place 250 µL of PI-PLC substrate (1 m*M* pyrene-PI) in a small test tube (10 × 75 mm) and dry under a stream of nitrogen and then under high vacuum for 10–15 min to remove all traces of solvent Add 200 µL of PI-PLC buffer. Vortex and sonicate (bath-type sonicator) repeatedly until the lipid is completely dissolved and the solution is clear
4. PI-PLC HPLC solvent 5 m*M* tetrabutylammonium dihydrogenphosphate (cat no 26,810-0, Aldrich, Milwaukee, WI) in acetonitrile/methanol/water (70 10.20)

2.3. HPLC Analysis

1 HPLC column for PLA_2 assay 15 cm × 4.6 mm, 5 µm spherical silica gel (cat no 85774, Waters, Milford, MA)—protect with a guard column.
2. HPLC column for PI-PLC assay· 25 cm × 4.6 mm 5 µm Spherisorb ODS (cat. no. 58312, Supelco, Bellefonte, PA)—protect with a guard column (*see* **Note 4**).
3. Fluorescence detector· Kratos model 980 or other suitable detector
4. HPLC instrument with autosampler (optional) and integrator/recorder

3. Methods

3.1. PLA_2 Assay

1 Mix 40 µL of PLA_2 stock assay solution with 60 µL of enzyme (containing the equivalent of about 2 ng of pure snake venom PLA_2 (cat no 525150, Calbiochem, La Jolla, CA) (*see* **Note 5**) in a 500-µL microcentrifuge tube (vortex) Incubate at room temperature. The final concentrations are 0.15 *M* NaCl, 0.1 m*M* substrate, 0 2 m*M* Triton X-100, 5 m*M* $CaCl_2$, 25 m*M* Tris-HCl, pH 7 0
2 At various times over a period of 30–60 min, remove 5-µL aliquots Spot directly on a TLC plate for TLC analysis, or add to 90 µL of quenching solvent in a 500-µL microcentrifuge tube for HPLC analysis (vortex)
3 For TLC analysis. dry the spots and develop the TLC plates in TLC solvent After the solvent has evaporated, scan the plates with a fluorescence densitometer (set excitation at 256 nm; measure emission above 400 nm [cutoff filter]). The R_f values for dansyl-PAF and lyso-dansyl-PAF are 0 35 and 0 15, respectively
4 For HPLC analysis centrifuge the quenched samples at top speed in a microcentrifuge for several minutes to remove any precipitated protein Equilibrate the silica gel HPLC column in PLA_2 HPLC solvent at a rate of 1 mL/min Set the fluorescence detector at excitation 256 nm and emission >418 nm (cutoff filter). Inject 20 µL of sample onto the column. Elution times for dansyl-PC and lyso-dansyl-PC are about 6 and 19 min, respectively
5 Calculation of activity. the mol fraction of product released is determined by dividing the area of the lyso-dansyl-PC peak by the sum of the areas of the dansyl-

PC and lyso-dansyl-PC peaks This value times the initial amount of substrate present in the assay (0 01 μmol) equals the amount of product released Plot the μmol of product released vs time to determine the initial linear rate (activity, μmol/min)

3.2. PI-PLC Assay

1 Add 5 μL of PI-PLC (containing the equivalent of 0 2–4 ng of pure enzyme (cat no P-6466, Molecular Probes) (*see* **Notes 5** and **6**) to 20 μL of PI-PLC stock assay solution in a 500-μL microcentrifuge tube (vortex). Incubate at room temperature Final concentration of substrate· 1 m*M*
2 At various times over a period of 10–30 min remove 5-μL aliquots and dilute with 95 μL of PI-PLC HPLC solvent in 500-μL microcentrifuge tubes (vortex) Centrifuge these samples at top speed in a microcentrifuge for several minutes to remove any precipitated protein (*see* **Note 7**)
3 Equilibrate the ODS reverse-phase HPLC column in HPLC solvent at a rate of 1 mL/min (*see* **Note 4**) Set the fluorescence detector at excitation 343 nm and emission >370 nm (cutoff filter) Inject 20 μL of sample onto the column. Elution times for pyrene-PI and pyrenebutanol are 2 4 and 6.2 min, respectively
4 Calculation of activity the mol fraction of product released is determined by dividing the area of the pyrenebutanol peak by the sum of the areas of the pyrene-PI and pyrenebutanol peaks. This value times the initial amount of substrate present in the assay (0 025 μmol) equals the amount of product released Plot the μmol of product released vs time to determine the initial linear rate (activity, μmol/min)

4. Notes

1 Derivatives of dansyl-PC are useful in the assay of other enzymes of lipid metabolism Lyso-dansyl-PC and dansyl-PAF (cat. no D-3766 and D-3767, respectively, Molecular Probes) can be used as substrates in assays of lyso-PAF acyltransferase, lyso-PAF acetyltransferase, and PAF acetylhydrolase *(3)*
2 This solution is stable for a year or more if protected from moisture and stored in a brown bottle at –20°C Before use, warm to room temperatuie and make sure the lipid is completely dissolved
3 Snake venom PLA_2 is quite active in the presence of Triton X-100, but other PLA_2s (particularly pancreatic PLA_2) may be less active with this detergent. The pancreatic enzyme is best assayed using sodium cholate as a detergent Hexadecylphosphocholine (cat no H6722, Sigma) is also a good detergent for other phospholipases. The concentration of substrate and the ratio of substrate to detergent may be varied as desired The specific activity of pure snake venom PLA_2 in this assay with dansyl-PC is about 13 μmol/min/mg
4 Do not leave the reverse-phase ODS column in PI-PLC HPLC solvent (with tetrabutylammonium dihydrogen phosphate) for any length of time without solvent running through; if so, the column will become plugged After use, immediately wash the column with CH_3OH/H_2O (80·20)

5 Phospholipases readily adsorb onto glass or plastic surfaces Dilute solutions of PLA_2 and PI-PLC (<1 mg/mL) should be stabilized by the presence of 1% (w/v) bovine serum albumin
6 With pure *B cereus* PI-PLC, the specific activity in this assay with pyrene-PI is about 60 μmol/min/mg, about 4 % hydrolysis is observed in 1 min with 4 ng of pure enzyme
7 R_f values for TLC of pyrenebutanol and pyrene-PI on silica gel plates 0.75 and 0 0, respectively, in $CHCl_3/CH_3OH$ (95 5), 1 0 and 0 25, respectively in $CHCl_3/CH_3OH/H_2O$ (65 35 3)

References

1. Reynolds, L J., Washburn, W N., Deems, R. A., and Dennis, E A (1991) Assay strategies and methods for phospholipases *Methods Enzymol* **197,** 3–23
2 Hendrickson, H S (1994) Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes *Anal Biochem* **219,** 1–8
3. Schindler, P W , Walter, R., and Hendrickson, H S (1988) Fluorophore-labeled ether lipids Substrates for enzymes of the PAF cycle in peritoneal polymorphonuclear leukocytes *Anal Biochem* **174,** 477–484
4 Hendrickson, H S , Kotz, K J , and Hendrickson, E K (1990) Evaluation of fluorescent and colored phosphatidylcholine analogs as substrates for the assay of phospholipase A_2 *Anal Biochem* **185,** 80–83
5 Hendrickson, H. S. (1991) Phospholipase A_2 assays with fluorophore-labeled lipid substrates *Methods Enzymol* **197,** 90–94
6 Bruzik, K S and Tsai, M -D (1994) Toward the mechanism of phosphoinositide-specific phospholipase C *Bioorg Med Chem* **2,** 49–72
7 Low, M G and Saltiel, A R (1988) Structural and functional roles for glycosylphosphatidylinositol in membranes. *Science* **239,** 268–275
8 Hendrickson, E K , Johnson, J L., and Hendrickson, H S (1991) A fluorescent substrate for the assay of phosphatidylinositol-specific phospholipase C 4-(1-pyreno)butylphosphoryl-1-*myo*-inositol *Bioorg Med Chem Lett* **1,** 619–622
9 Hendrickson, H S , Hendrickson, E K , Johnson, J L , Khan, T H , and Chial, H J (1992) Kinetics of *B cereus* phosphatidylinositol-specific phospholipase C with thiophosphate and fluorescent analogs of phosphatidylinositol *Biochemistry* **31,** 12,169–12,172