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High-Throughput Assay of Secreted Phospholipases A₂ Inhibitors

Wallace Thompson, Rob C. Oslund, James Bollinger, Heather Ewing, and Michael H. Gelb

Abstract

Attempts to characterize, quantify, and/or modulate the activity of the secreted phospholipase A₂ family of enzymes result from the diversity of physiological roles for which these enzymes have been implicated. The 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyrenePG)-based fluorometric assay is a sensitive and readily adaptable method for further elucidating phospholipase function under various experimental conditions, as well as a tool for screening chemical libraries for potent inhibitors of this enzymatic activity. This assay is based on the observed difference in fluorescent emission of pyrene aggregated in vesicles compared to sequestered in monomeric form by binding to bovine serum albumin after lipolytic activity, thus allowing direct quantification of hydrolyzed fatty acids by the measurement of the corresponding monomeric emission intensity. The assay can be carried out in multiwell plates for high-throughput screening of compound libraries.

Keywords

Phospholipase A₂; Fluorometric assay; High-throughput screen; Interfacial enzymology; PLA₂; Fluorogenic; 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol; Secreted PLA₂ Inhibition Assay

1. Introduction

Phospholipases A₂ (PLA₂s) constitute an enzyme family that catalyzes the hydrolysis of the *sn*-2 ester bond of phospholipid substrates, producing free fatty acids and lysophospholipids (1). Physiological roles of PLA₂s have been shown to include the hydrolysis of esterified arachidonyl acyl chains from many distinct phospholipid membranes in a variety of tissues (2–4). This catalytic activity liberates free arachidonic acid, a precursor of prostaglandins, prostacyclins, leukotrienes, and other inflammatory-mediating eicosanoids created by downstream modification (2, 5–7).

One early attempt to design a fluorescence-based phospholipase A₂ activity assay made use of a pyrenobutanoyl acyl chain at the *sn*-2 position of phosphatidylcholine (8). Later, phospholipid substrates with 10-pyrenedecanoyl acyl chains at the same position came into use (9). This chapter describes a similar assay with 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyre-nePG) as a substrate. A common aspect of these assays is that they are all based upon the varying emission maxima of pyrene excimers in vesicular form

as opposed to monomeric pyrene sequestered by binding to excess bovine serum albumin (BSA) present in the aqueous phase. Only after the release of 10-pyrenedecanoic acid from the phosphatidylglycerol does the pyrene fluorophore desorb from the membrane vesicles to bind to BSA in the aqueous phase. By following the fluorescence of monomeric 10-pyrenedecanoic acid bound to albumin, the activity of phospholipases A₂ can be measured in real time.

2. Materials

2.1. Fluorescent Product and Substrate

2.1.1. 10-Pyrenedecanoic Acid (see Subheading 3.1)

1. C16:0LysoPG(1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), Avanti, cat no. 858122P).
2. Acetone.
3. Round bottom flask.
4. 2,2-Dimethoxypropane.
5. Trifluoroacetic acid (CF₃COOH).
6. CHCl₃.
7. Methyl 10-chloro-10-oxodecanoate.
8. AlCl₃.
9. Pyrene.
10. Ice bath.
11. Separatory funnel.
12. HCl.
13. Vacuum distiller/Rotary evaporator.
14. Silica gel for flash chromatography.
15. Diethylene glycol (DEG).
16. Hydrazine hydrate (H₂NNH₂).
17. NaOH.
18. Nitrobenzene.
19. Reflux equipment.

2.1.2. PyrenePG (See Subheading 3.1 and Fig. 1)

1. 4-Dimethylaminopyridine (DMAP).
2. Triethylamine (NEt₃).
3. Dry chloroform (CHCl₃).

4. 2,4,6-Trichlorobenzoyl chloride.
5. H₂O.
6. MeOH/CHCl₃ solution: 20% methanol, 80% CHCl₃(v/v).
7. Silica gel for flash chromatography.
8. AcOH solution: 80% acetic acid in H₂O.
9. Na₂SO₄.
10. Separatory funnel filter.
11. Round bottom flask.

2.2. Working Stock Solutions, Assay Buffer, and Experimental Solutions

This phospholipase A₂ assay takes place in a buffer imitating physiological conditions which may be made in large amounts and stored at 4°C for months. Three solutions—buffer with BSA, enzyme, or substrate—should be made from this original assay buffer daily before undertaking any experiments.

1. PyrenePG working solution: 100 μM pyrenePG (see Subheading 3.1) in 200 proof ethanol. Stored at −20°C and quantified before each use.
2. Assay buffer: 50 mM KCl, 1 mM CaCl₂, 50 mM Tris-HCl, pH 8.0. Filtered through a 0.22 μm sterile filter and stored at 4°C. Each well measured requires 300 μL of this buffer.
3. BSA solution: 3% fatty acid-free BSA (see Note 1) in assay buffer. Filtered through a 0.2 μm cellulose acetate sterile syringe filter, aliquoted, and stored at −20°C. Each 970 μL of assay buffer requires 30 μL of this solution before use.
4. Solution A: 30 μL BSA solution added to 970 μL assay buffer. Each well measured requires 100 μL of this solution.
5. Solution B: appropriate enzyme amount added to Solution A. Each well measured requires 100 μL of this solution.
6. Solution C: pyrenePG working solution added to assay buffer (no BSA) to yield 4.2 μM pyrenePG. Each well measured requires 100 μL of this solution.
7. Phospholipase A₂ enzyme(s).

3. Methods

3.1. Synthesis of Fluorescent 10-Pyrenedecanoic Acid Product and pyrenePG Substrate (See Fig. 1)

3.1.1. Acetonide Protection of LysoPG—Procedures and tasks related to this assay include: synthesis of product and substrate (see Subheading 3.1) and calibration of fluorimeter (see Subheading 3.2).

¹Ensure that fatty-acid free BSA (e.g., Sigma A6003) is used.

After the initial synthesis of pyrenePG substrate and 10-pyrene-decanoic acid product, these chemicals may be stored indefinitely at -20°C for future use. The 10-pyrenedecanoic acid product is necessary for calibrating the fluorimeter response.

1. Suspend 185 mg, (0.36 mmol) of C16:0 LysoPG (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), Avanti, cat no. 858122P) in 3 mL acetone in a round bottom flask.
2. Add 170 mL of 2,2-dimethoxypropane to the reaction mixture followed by addition of a catalytic amount of 99% CF_3COOH (100 μL).
3. Also add CHCl_3 until the lysoPG is fully dissolved.
4. Stir the reaction mixture until full conversion to acetonide protected product [as measured by ESI-MS in the negative mode (product peak MW: 523.3, starting material peak MW: 483.5)]. Full conversion to product requires about 60 h stirring at room temperature.
5. Stop the reaction by removing solvent by rotary evaporation to give a dark orange oil.
6. Dry the oil in vacuo for 30 min to remove most of the CF_3COOH .
7. Dissolve the product in dry 10–15 mL CHCl_3 (do not use wet CHCl_3 as this will react with the acetonide moiety), and remove the solvent by rotary evaporation. Repeat three times to remove CF_3COOH in the oily residue.
8. Dry the oily residue in vacuo overnight to give an oil (274 mg, 150% excess weight is likely due to unremoved TFA). ^1H NMR (300 MHz, CDCl_3): 0.90 (t, $J = 6.3$ Hz, 3H), 1.27 (s, 25H), 1.37 (s, 3H), 1.44 (s, 3H), 1.61 (m, 3H), 2.34 (t, $J = 7.2$ Hz, 2H), 3.78 (m, 2H), 3.93–4.10 (m, 6H), 4.24–4.34 (m, 2H) MS (ESI neg. ion) m/z : 523.3 (M–1).

3.1.2. Methyl 10-oxo-10-(Pyren-3-yl)Decanoate

1. Dissolve methyl 10-chloro-10-oxodecanoate (265 μL , 2.0 mmol) in 4 mL nitrobenzene and stir at 0°C in an ice bath.
2. Add AlCl_3 (264 mg, 2.0 mmol) in portions over 1 min. After addition of all AlCl_3 , stir the reaction mixture for an additional 2–3 min and then add pyrene (200 mg, 0.99 mmol) to the reaction mixture (reaction turns blood red color).
3. Remove the ice bath and stir the reaction mixture for 18 h at room temperature.
4. Pour onto 5 mL ice cold water in a separatory funnel. Acidify the water layer with 1 N HCl, and separate the layers.
5. Extract the water layer with 3×5 mL CHCl_3 .
6. Combine the organic layers with the nitrobenzene layer.
7. Remove the CHCl_3 by rotary evaporation, and remove the nitrobenzene layer by vacuum distillation (6 mm Hg, 80°C).

8. Dissolve the yellow residue in 2–3 mL CHCl₃ and purify by flash chromatography over silica gel (100% CHCl₃) to give a yellow solid (250 mg, 63% yield). ¹H NMR (300 MHz, , CDCl₃): 1.33–1.46 (m, 8H), 1.61 (m, *J* = 7.5 Hz, 2H), 1.86 (m, *J* = 7.2 Hz, 2H), 2.29 (t, *J* = 7.5 Hz, 2H), 3.21 (t, *J* = 7.2 Hz, 2H), 3.65 (s, 3H), 8.03–8.33 (m, 8H), 8.85 (d, *J* = 10.8, 1H).

3.1.3. Synthesis of 10-(Pyren-3-yl) Decanoic Acid

1. Dissolve methyl 10-oxo-10-(pyren-3-yl)decanoate (see Subheading 3.1.2) (2.9 g, 7.3 mmol) in 10 mL DEG followed by addition of 65% hydrazine hydrate (1.95 mL, 40 mmol) and NaOH (2.3 g, 50 mmol).
2. Reflux the reaction mixture for 2 h at 190°C.
3. Replace the reflux condenser with a distillation apparatus and stir the reaction at 195°C for 30 min to remove any low boiling solvents formed during the reaction.
4. Cool the reaction mixture to room temperature, and attach again the reflux condenser to the flask.
5. Reflux overnight at 190°C.
6. Cool to room temperature and add 8 mL H₂O.
7. Add concentrated HCl to pH XXX make the reaction mixture acidic.
8. Collect the yellow precipitate formed by vacuum filtration.
9. Dry the yellow precipitate in vacuo overnight and purify by flash chromatography (95% CHCl₃/5% MeOH) to give a light yellow solid (1.5 g, 57% yield). ¹H NMR (300 MHz, CDCl₃): 1.31 (s, 8H), 1.48 (m, 2H), 1.62 (m, 2H), 1.85 (q, *J* = 7.8 Hz, 2H), 2.33 (t, *J* = 7.5 Hz, 2H), 3.33 (t, *J* = 7.8 Hz, 3H), 7.86 (d, *J* = 7.8 Hz, 1H), 7.95–8.17 (m, 7H), 8.28 (d, *J* = 9.3 Hz) MS (ESI neg. ion) *m/z*: 371.3 (M – 1).

3.1.4. Acetonide-Protected 1-Palmitoyl-2-(1-Pyrenedecanoyl)-sn-Glycero-3-Phosphatidylglycerol (Acetonide-Protected Pyrene PG)

1. Dissolve acetonide protected lysoPG (see Subheading 3.1.1) (244 mg, 0.45 mmol), 10-pyrenedecanoic acid (see Subheading 3.1.3) (415 mg, 1.1 mmol), 4-dimethylaminopyridine (330 mg, 2.7 mmol), and NEt₃ (220 μL, 1.6 mmol) in 15 mL dry CHCl₃ and stir at room temperature for 10 min.
2. Add 2,4,6-trichlorobenzoyl chloride (450 μL, 2.90 mmol) to the reaction mixture, and stir it overnight at room temperature.
3. After stirring overnight, add 500 μL H₂O to the reaction mixture to quench the acid chloride, and remove the solvent by rotary evaporation to give a crude oil.
4. Dissolve the crude oil in 2 mL MeOH/CHCl₃ solution and purified by column chromatography over silica gel (20% MeOH/80% CHCl₃).
5. Dry the product in vacuo to give a white/brown solid (190 mg, 48% yield). MS (ESI neg. ion) *m/z*: 878(M-1).

3.1.5. 1-Palmitoyl-2-(10-Pyrenedecanoyl)-sn-Glycero-3-Phosphatidylglycerol (pyrenePG)

1. Dissolve acetone-protected pyrenePG (see Subheading 3.1.4) (190 mg, 0.21 mmol) in 1 mL CHCl_3 .
2. Add of 5 mL 80% acetic acid in H_2O .
3. Stir the reaction mixture for 1 h at 60°C .
4. Cool the reaction mixture to room temperature and remove the solvent by rotary evaporation.
5. Dissolve the crude amber solid in 2 mL $\text{MeOH}/\text{CHCl}_3$ solution and purify by column chromatography over silica gel (20% $\text{MeOH}/80\% \text{CHCl}_3$).
6. Collect the fractions containing product and remove the solvent by rotary evaporation.
7. Redissolve the product in 4 mL CHCl_3 and pour into a separatory funnel containing 20 mL H_2O and 20 mL CHCl_3 .
8. Pour the product into the separatory funnel over a cotton plug to remove any trace silica.
9. Wash the product with $2 \times 20 \text{ mL } \text{H}_2\text{O}$.
10. Dry the layers separated over Na_2SO_4 and filter into a round bottom flask.
11. Remove the solvent to give an amber/white solid (74% yield). ^1H NMR (300 MHz, CDCl_3): 0.83 (m, 3H), 1.17–2.0 (m, 43H), 2.26 (m, 4H), 3.22 (m, 2H), 3.60–4.40 (m, 9H), 5.25 (m, 1H), 7.76–8.16 (m, 9H) MS (ESI neg. ion) m/z : 838.1 ($\text{M} - 1$).

3.1.6. PyrenePG Conservation

1. Dissolve dried pyrenePG in CHCl_3 at 1 mg/mL concentration.
2. Transfer 1 mL of the solution to an amber screw-cap vial and remove the solvent either in a centrifugal concentrator (Speed-vac) or by passing a stream of N_2 into the vial to give a dried powder.
3. Store the dried powder ($\approx 1 \text{ mg/vial}$) in a storage jar at -80°C with desiccant. Typically, 1 mg of pyrenePG is enough to make about 90 mL of $4.2 \mu\text{M}$ pyrenePG for the assay.

3.2. Calibration of Fluorimeter

Following the procedures described in the rest of the methods section below, prepare and measure wells containing pyrenedecanoic acid in amounts similar to those expected to be obtained during enzymatic activity assays. Use the resulting fluorescent readout to create a standard curve for the fluorometric instrument. For our purposes, excitation and emission wavelengths of 342 and 405 nm, respectively, provided maximum sensitivity (see Note 2).

3.3. Preparation of pyrenePG Working Solution from dry pyrenePG Stock

1. Dissolve approximately 1 mg pyrenePG in 1 mL 1:1 toluene:ethanol in a round-bottomed glass culture tube.
2. Vortex and sonicate extensively to resuspend the reagent, using hand warmth to facilitate the process. Sonication could be performed with a bath sonicator (e.g., Laboratory Supplies Co., Inc., Hicksville, NY; 80 watt output; model G112SP1G). Avoid sonication of flat bottom vials containers as this may lead to shattering.
3. Remove the solvent by centrifugal evaporation (Speed-Vac), and resuspend the compound again in 2.5 mL pure ethanol, vortexing, sonicating, and heating with hands to aid the process as before.
4. Transfer the solution to two 1.5 mL microtubes, centrifuging to remove insoluble particles, and remove the supernatant for use in the assay.

3.4. Quantification of pyrenePG Working Solution

Determining the amount of active pyrenePG is necessary due to possible incomplete resuspension of the reagent in ethanol.

1. For calibration, use 1 mL of pure ethanol as a blank while measuring the absorbance at 342 nm.
2. Add 20 μ L of pyrenePG working solution and measure again, applying the appropriate dilution factor to the absorbance measurement.
3. Repeat with differing amount of substrate to ensure a linear response if desired. The molar extinction coefficient of pyrenePG is approximately 40,000 cm^{-1} .

3.5. Optimization of Enzyme Amount Used per Well

The amount of enzyme used in each well should be enough to ensure a fluorescent change of at least ten times background. Therefore, care should be taken to first obtain a consistent value of background fluorescence change in the absence of enzyme: this number is usually significant and cannot be assumed zero. Next, the minimum amount of enzyme necessary to produce a monomer fluorescence emission of at least ten times background should be determined through trial and error (see Notes 3 and ⁴).

²Varying slightly the excitation and emission wavelengths may increase sensitivity on other instruments; the wavelengths provided are optimal for our purposes. Measuring emission wavelengths significantly above 400 nm risks exposure to the fluorescent emission of pyrene excimers. The fluorimeter used in our laboratory is a Victor³ V Model 1420-040 by Perkin Elmer, Inc. All assays were performed at room temperature (21°C) with ten 1.0 s measurements made per well. The excitation and emission filters used were P340 (10 nm bandwidth) and D405 (10 nm bandwidth), respectively.

³Over the course of several assays, e.g., high-throughput screening, fluorescence change may decrease. This is usually due to actual attenuation of enzymatic activity; therefore, it behooves the scientist to use fresh enzyme dilutions as often as possible, keep these solutions on ice, store pyrenePG substrate in darkness, and/or intermittently measure fluorescence change values of positive control wells to use in data analysis. PyrenePG vesicles are stable on the benchtop for at least several hours.

⁴Addition of even small amounts of organic solvents or detergents can drastically alter this assay through vesicle disruption. The pyrenePG stock concentration must be greater than 100 μ M to prevent introduction of excessive amounts of ethanol. Organic solutions of inhibitors or compounds to be screened may lead to similar effects. Seemingly innocuous sources of contamination, such as detergents, present in enzyme stock solutions may have severely detrimental effects even at low concentration.

3.6. Addition of Solutions to 96-Well Plate

Since this assay ultimately measures fluorescence change over a short time period, care should be taken to ensure simultaneous reaction initiation in each well. This can be accomplished by using a multi-channel micropipette or other high-throughput device to add substrate to wells already containing enzyme, an effective method since the large volume (100 μ L; one-third of total well volume) added thoroughly mixes the well contents (see Notes 5 and 6).

For clarity, the order of addition of each experimental solution for control and inhibition wells is enumerated here:

Negative Control Well: 200 μ L Solution A followed by 1 μ L DMSO and 100 μ L Solution C.

Inhibition Screen Well: 100 μ L Solution A, 1 μ L DMSO with inhibitor (see Note 4), 100 μ L Solution B, 100 μ L Solution C.

Positive Control Well: 100 μ L Solution A, 1 μ L DMSO, 100 μ L Solution B, 100 μ L Solution C.

3.7. Other Assays Using pyrenePG

Due to its ease of use, the pyrenePG assay may be used for a variety of purposes other than high-throughput screens (see Note 7).

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⁵Alternatively, calcium may be added for the initiation of calcium-dependent enzymatic reactions. In this case, assay buffer lacking calcium should be made and used in the preparation of all solutions. Addition of a calcium chelator, such as EGTA, will ensure the absence of free calcium from the reaction mixture if desired, and enough calcium should be added in excess of the chelator at the initiation time point.

⁶Since some fluorimeters measure the fluorescence signal from each well separately and serially, the overall elapsed time of different assays may vary depending on the total number of wells included. This often must be considered when making direct comparisons of experiments, and the best way to avoid any discrepancies is to measure the same number of wells during every experimental trial.

⁷Examples include direct kinetic comparison of phospholipase activity on mixed vesicles, quantification of stock solutions of enzymes with predetermined velocities, determining the extent of overall enzymatic inhibition by small molecules or of active site-specific binding by potential active site inhibitors (10), and quantification of pyrenePG-containing vesicles for use in other assays. Additionally, one could conceivably synthesize pyrenePG substrates with the fluorescent moiety present at the *sn*-1 position for quantification of PLA1 activity.

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Fig. 1. Synthesis of 1-palmitoyl-2-(10-pyrenedecanoyl)-phosphatidylglycerol (pyrenePG). **(a)** 2,2-dimethoxypropane, CF_3COOH , acetone, rt, 60 h. **(b)** AlCl_3 , nitrobenzene, rt, 18 h, 63% yield **(c)** H_2NNH_2 , NaOH , DEG, 195°C , 6 h, 57% yield. **(d)** DMAP, Et_3N , CHCl_3 , rt, 3 h, 48% yield. **(e)** $\text{AcOH}/\text{H}_2\text{O}$, CHCl_3 , 60°C , 1 h, 74% yield.