

High throughput phosphoglycolate phosphatase activity assay using crude leaf extract and recombinant enzyme to determine kinetic parameters K_m and V_{max} using a microplate reader

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Abstract

Determining enzyme activities involved in photorespiration, either in a crude plant tissue extract or in a preparation of a recombinant enzyme, is time-consuming, especially when large number of samples need to be processed. This chapter presents a phosphoglycolate phosphatase (PGLP) activity assay that is adapted for use in a 96-well microplate format. The microplate format for the assay requires less enzyme and reagents and allows rapid and less expensive measurement of PGLP enzyme activity. The small volume of reaction mix in a 96-well microplate format enables determination of PGLP enzyme activity for screening many plant samples, multiple enzyme activities using the same protein extract, and/or identifying kinetic parameters for a recombinant enzyme. To assist in preparing assay reagents, we also present an R Shiny buffer preparation app for PGLP and other photorespiratory enzyme activities and a K_m and V_{max} calculation app.

Keywords

Photorespiration, phosphoglycolate phosphatase, recombinant phosphoglycolate phosphatase, buffer preparation app, Michaelis–Menten, K_m and V_{max} calculation app

1 Introduction

Rubisco-catalyzed oxygenation of ribulose-1,5-bisphosphate (RuBP) initiates the photorespiratory pathway (PR) with the formation of 2-phosphoglycolate (2-PG) which is inhibitory to C3 cycle enzymes and hence net CO₂ assimilation. Phosphoglycolate phosphatase (EC 3.1.3.18) (PGLP) is the “first line of defense” to protect the cell from inhibitory 2-PG by hydrolyzing 2-PG into glycolate and phosphate. This first line of defense is critical due to the scale of rubisco oxygenation, for instance, in all photosynthesizing organisms on earth rubisco is involved in sequestering ~100 billion tons of oxygen compared to fixation of ~250 billion tons of carbon dioxide annually [1].

During the multistep process of PR, which involves ~10 core enzymes located in chloroplasts, peroxisomes, and mitochondria, 2-PG is processed and its carbon returns to the C3 cycle in the form of 3-phosphoglyceric acid (3-PGA). PGLP catalyzes the first reaction in PR downstream from rubisco and is essential for photosynthetic growth at ambient CO₂ [2, 3]. Furthermore, overexpression of PGLP resulted in enhanced tolerance of *A. thaliana* to high light, drought, and high temperature [4]. Moreover, *Rhazya stricta*'s particularly high level of photosynthetic and PR activity at elevated temperature, is associated with a higher activity of PGLP (Gregory et al., 2023).

Here we provide a method for measuring PGLP activity in a 96-well format suitable for high-throughput enzyme activity measurements in crude cell extract from leaf tissue and preparation of purified recombinant PGLP, a procedure for obtaining purified recombinant 6xHis-AtPGLP expressed in *E. coli*, and a protocol outlining steps for determining K_m and V_{max} of a purified recombinant AtPGLP using these approaches.

2 Materials

2.1 Plant material. This approach can be used with any plant material that can be homogenized but there will likely be some optimization needed based on the activity of the specific samples. For example, we use *Nicotiana tabacum* grown in the greenhouse at ~30 °C until plants formed 5-7 fully developed leaves. In the protocol described below we provide specifics for measuring PGLP activity using *N. tabacum* leaves. However, the PGLP activity measurement was successfully accomplished in our laboratory using the outlined protocol in the leaf material from *Rhazya stricta* (*Rhazya* was grown for ~1.5 years and sampled with six 8.15 mm diameter punches) and birch (at least 1-year-old and approximately 120 mg of frozen leaf tissue).

2.2 Total Equipment list

1. Cork borer, ~17 mm diameter
2. 2 ml screw cap microcentrifuge tubes
3. 1.5 ml snap cap microcentrifuge tubes
4. Microplate reader
5. 96-pin microplate replicator (e.g., Boekel Scientific, cat. # 140500)
6. Refrigerated bench-top centrifuges which work with sample sizes of 0.2-2.0 ml and 15-50 ml
7. Swinging bucket bench-top centrifuge with adapter for 96-well plate
8. Pipettes, 2 ul, 20 ul, 300 ul, 1 ml
9. Multichannel pipette, 300 ul
10. Tips for pipettes 10 ul, 200 ul, 300 ul, 1 ml
11. 2 ml glass-to-glass homogenizer (e.g., Kontes Glass Co., Vineland, NJ, USA)
12. Reservoir for multichannel pipet (e.g., Thomas Scientific)

13. Polystyrene 96-well plates
14. Digital Timer
15. Parafilm
16. Oven at 104 °C
17. Volumetric flask 100 ml
19. 15 ml and 50 ml plastic tubes
20. Glass tubes 16 x 125 mm
21. Autoclavable culture tube caps, plastic, or metal
22. Sterile toothpicks, pointed.
23. Incubator with orbital shaking platform for 37 °C and 25 °C
24. Ni-NTA Spin columns
25. Sonicator
26. Benchtop liquid nitrogen container
27. Biosafety cabinet

2.3 Reagents and solutions

Use ultrapure sterilized water for all solutions.

2.3.1 PGLP activity measurement

1. Extraction buffer (50 mM EPPS buffer, pH 8.0, containing 1 mM EDTA, 10 mM DTT, 0.1% Triton X-100 [v/v], 0.5% polyvinylpyrrolidone, and 10 µl 1X protease inhibitor cocktail, EDTA free. 50 mM EPPS, pH 8.0, and stock solutions 0.5 M EDTA, 1 M MgCl₂, and 4 M KCl should be autoclaved and stored at room temperature. Prepare extraction buffer fresh

before use. Refer to the buffer calculation app (https://github.com/L-Gregory/PhotorespiratoryAssay_Recipes) for amounts of chemicals needed to prepare extraction and reaction buffers; Figure 1.

2. Reaction buffer 50 mM HEPES buffer, pH 7.5, 1 mM EDTA, and 10 mM MgCl₂. 50 mM HEPES, pH 7.5, can be prepared in advance, filter-sterilized, and stored at +4 °C. 1 M MgCl₂ stock solution should be autoclaved and stored at room temperature. Prepare reaction buffer fresh before use.
3. Phosphate stock KH₂PO₄ 50 µg/ml. Dehydrate KH₂PO₄ for 1.5-2 h at 104 °C, store in a desiccator before use. The stock can be stored in a tightly closed flask at 4 °C for at least 1 month.
4. 200 mM 2-phosphoglycolic acid lithium salt (2-PG). Aliquot by 0.1 ml, store at -80 °C.
5. Reagent for phosphate determination: 2.5 N sulphuric acid, 0.2 mM antimony potassium tartrate, 4.9 mM ammonium molybdate tetrahydrate, and 30.3 mM ascorbic acid. Prepare the following stocks: the 5 N sulphuric acid stock can be stored at room temperature indefinitely, 4 mM antimony potassium tartrate trihydrate and 32.7 mM ammonium molybdate tetrahydrate can be stored at room temperature for up to a month, and the 100 mM ascorbic acid should be prepared fresh. To make 10 ml of the reagent, mix the solutions before application in the following quantity: 5 ml 5 N sulfuric acid, 0.5 ml 4 mM antimony potassium tartrate, 1.5 ml 32.67 mM ammonium molybdate tetrahydrate, and 3 ml 100 mM ascorbic acid.

2.3.2. Expression and purification of recombinant AtPGLP

1. Kanamycin 50 mg/ml stock, aliquot and store at -20 °C.

2. Sterile LB medium, liquid.
3. Sterile LB containing 15 g/L agar and 50 µg/ml kanamycin in Petri dishes.
4. 80% sterile glycerol.
5. 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside), filter through a sterile 0.22 µm filter using a syringe, aliquot, store at -20 °C.
6. 50 mM Tris-HCl, 300 mM NaCl, pH 8.0. You may prepare the buffer a day before, store at +4 °C.
7. Lysis buffer: 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNase1, 10 mM CaCl₂, proteinase inhibitors, 10 mM imidazole, 5% glycerol.
Prepare lysis buffer fresh before use.
8. Buffers containing imidazole for binding, washing and elution prepare fresh, add imidazole to buffers just before use, keep on ice. Binding buffer 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole. Washing buffer 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole. Elution buffer 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole.

2.3.2 Determining Protein concentration

1. Bradford 1x Dye Reagent, store at +4 °C. Bring to room temperature an aliquot necessary for construction the standard curve and processing the number of leaf extracts for a particular experiment.
2. Bovine serum albumin (BSA) dilution series of standards (2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml), store at -80 °C.

Methods

3. Preparing crude protein extract.

1. For one sample, collect three *N. tabacum* leaf punches each 17 mm in diameter (total area=6.81 cm², approximately 120 mg) (may be more or less depending on species as discussed in Materials section) from fully developed uppermost leaves located next to each other. Using clean forceps quickly place leaf punches into a labeled 2 ml screw cap plastic tube, freeze immediately in liquid N₂, and store at -80 °C. Do not allow to thaw until analysis.
2. Transfer the frozen leaf punches into a glass homogenizer kept on ice, add 1 ml cold extraction buffer, and homogenize manually for approx. 3-5 min until green is removed from the tissues, indicating cells have been ruptured.
3. Transfer the homogenate into a 1.5 ml plastic tube and clarify by centrifugation for 10 min at 13,500 g, +4 °C. The supernatant represents the crude protein extract and is used in the enzyme assay immediately.

4 Expression and purification of a recombinant *Arabidopsis thaliana* PGLP (AtPGLP).

Expression of recombinant N-terminal 6xHis tagged AtPGLP containing no targeting peptide is performed using NEB T7 express *E. coli* cell line transformed with pET28a(+)-AtPGLP.

The plasmid is available upon request. Under described below conditions at least a part of the recombinant protein is soluble according to the SDS-PAGE data. The procedure outlined below results in 90-95% and higher purity of isolated recombinant protein.

4.1 Expression

1. Inoculate 3 ml of sterile B containing kanamycin (50 µg/ml) in a glass tube (16x125 mm) with a single colony from agar plate (LB+Kan) using a sterile toothpick or sterile 10 µl tip; perform these procedures in a biosafety hood. Cover the tube with a culture tube cap. Grow the culture at 37 °C overnight at 225 rpm.
2. The next day, inoculate 50 ml in 250 ml flask of pre-warmed sterile LB media containing kanamycin (50 µg/ml), with 0.5 ml of the overnight cultures and grow at 37 °C with vigorous shaking until reaching an OD₆₀₀ of 0.6-0.9 (~2.5 h).

Take 1 ml sample in 1.5 ml plastic tube immediately before induction, this sample is to check expression in the non-induced culture. Pellet cells for 10 min at 3500 x g and +4 °C, discard supernatant, freeze the cell pellet on dry ice, store at -20 °C until analysis by SDS-PAGE.

3. then freeze in liquid N₂ and store at -20 °C until analysis by SDS-PAGE.
4. Pre-cool cultures to 25 °C (~5 -10 min). Add IPTG to a final concentration of 0.1 mM (50 µl 1 M IPTG/50 ml culture) to induce expression.
5. Incubate the cultures overnight at 25 °C until the OD reaches ~ 2. Collect a second 1 ml sample for the induced culture and pellet cells for 10 min at 3500 x g and +4 °C, then freeze in liquid N₂ and store at -20 °C until analysis by SDS-PAGE.
6. Harvest the cells from the 50 ml culture by centrifugation at 3500 x g for 20 min and +4 °C in 50 ml plastic centrifuge tube.
7. Completely remove supernatant and use the pelleted cells for purification immediately or freeze the cells in liquid N₂ and store cell pellet at -80 °C.

4.2 Purification

Binding, washing, and elution are basically performed according to the manufacturer's Handbook [5] with modifications outlined below. Carry out all steps at +4 °C. Perform purification procedure using 50 mM Tris-HCl, pH 8.0, as K-phosphate buffer, recommended by most manufacturers, interferes with the downstream detection of phosphate used for PGLP activity measurement.

1. Prepare a Ni-NTA column as recommended in the manufacturer's Handbook with modifications: apply 600 µl of 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole to the column and centrifuge the column at 890 g for 2 min and +4 °C.
2. Thaw the cell pellet for approx. 15 min on ice. Add 1.5 ml lysis buffer to cells obtained from 50 ml induced culture. Mix thoroughly the bacterial pellet and the lysis buffer. (See Note 1)
3. Incubate the combined cell suspension for 1 h on ice with gentle shaking.
4. Keeping the tube with the cell suspension on ice, sonicate 5x10 sec using a sonicator at high frequency with 10 sec intervals for cooling.
5. Centrifuge 20 min, 14,500 rpm, +4 °C (centrifugation could be performed in 1.5-2 ml plastic tubes).
6. Collect the supernatant (this is crude soluble protein extract) into 15 ml conical tube, keep on ice.
7. Apply 600 µl of the supernatant onto a column placed into a 2-ml collection tube and centrifuge the column with the tube at 50 g for 2 min at +4 °C. The low speed provides time for the enzyme to bind the Ni-matrix. Discard the flow-through but collect 50 µl and store at -20 °C until analysis by SDS-PAGE.
8. Repeat step 9 until all supernatant has been loaded.

9. Wash 3 times by applying 600 μ l 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole followed by centrifugation at 890 g for 2 min at +4 °C. Discard washes but collect 50 μ l of washes and store at -20 °C until analysis by SDS-PAGE.
10. Elute the enzyme by applying 300 μ l 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole to the column followed by centrifugation at 890 g for 2 min at +4 °C.
11. Repeat step 12.
12. Pool the eluates (you may keep the eluates separately if there are differences in purity) and dialyze twice against 250 ml 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl at +4 °C, then overnight against 1000 ml 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 5% glycerol at +4 °C (See Note 2).
13. The next day, if protein precipitation is present, remove the precipitated protein by centrifugation at 14,000 rpm and +4 °C for 10 min.
14. Measure protein concentration in the supernatant using NanoDrop and/or Bradford protocol for microplate (See below).
15. Add 80% sterile glycerol to result in a final 10% concentration to the dialyzed protein preparation, aliquot, and store at -80 °C.
16. Using SDS-PAGE (not provided in this Chapter) analyze the non-induced and induced cultures, crude soluble extract, flow-through, washes, and eluates.

5 Phosphoglycolate phosphatase (PGLP) activity. Methods for PGLP assay in different photosynthesizing species reported in literature are based on detection of liberated inorganic

phosphate during the enzymatic reaction. However, concentrations of the substrate 2-PG, MgCl_2 , pH, temperature, the buffer used and the assays for phosphate detection were different [6–10]. Here, we describe an assay to measure PGLP activity based on publication by Schwarte and Bauwe [11], with modifications. The pH of PGLP reaction is selected at pH 7.5 based on a report by J. Shen [12] that estimates pH in the plastid stroma at 7.2 ± 0.3 . Formation of inorganic phosphate is determined by a procedure published previously [13].

1. Before the assay, dilute the phosphate stock 10 times in 50 mM HEPES buffer, pH 7.5, to obtain a diluted stock 5 $\mu\text{g P/ml}$. Pipette the diluted KH_2PO_4 stock solution to produce 0.00, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, and 0.35 $\mu\text{g P/ml}$ per well in triplicate with final volume of 200 μl (Table 1). These samples are used for the standard curve construction.
2. Pipette 194 μl of reaction buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl_2) into empty wells in triplicate using a 300 μl multichannel pipette and add 4 μl of crude protein extract into each of these wells.
3. Make blank/background well: to 200 μl 50 mM HEPES, pH 7.5.
4. In case of recombinant enzyme, instead of crude protein extract, add 4 μl of diluted purified enzyme containing 10 μg of protein.
5. Initiate the enzymatic reaction by adding 2 μl of 200 mM 2-PG using the 96-pin replicator and mix thoroughly. 2 μl of the substrate can be added on the top or the side of the pins of the replicator to accomplish the substrate addition as simultaneously as possible. Figure 2.
6. Incubate the reaction mix for 5 min at 25 °C.
7. Terminate the reaction by adding 32 μl of reagent using a 100 μl multichannel pipette. Add the reagent to the blank/background well.

8. Cover the plate with the parafilm, keep the plate at room temperature, and take absorbance readings after 45 min at 880 nm using a microplate spectrophotometer. (Notes 3, 4, 5).
9. Make a control to adjust for phosphate that is not produced during the enzymatic reaction. For the control, combine in the following order: 194 μ l reaction buffer, 4 μ l crude protein extract, incubate for 5 min at 25 °C, then add 32 μ l of reagent to stop any enzymatic reactions followed by addition of 2 μ l of 200 mM 2-PG.
10. Determine phosphate amounts using the spectrometer's software to plot standard curve (Figure 2) and calculate amounts of phosphate produced in the wells containing enzymatic reaction mixes and the control wells. Plate reader software will automatically subtract the blank/background OD value from OD values in all read wells. Subtract the control values from the corresponding values determined in the wells with enzymatic reaction mixes (Figure 3).

6 Protein concentration is determined by the Bradford method [14] using a microplate reader . The assay is based on binding Coomassie Brilliant Blue G-250 dye, a component of Bradford 1x Dye Reagent to protein and formation of the blue color product under acidic conditions with the absorbance maximum at 595 nm. The assay is performed in a standard 250 μ l microplate assay format with using BSA in the linear range 125–1,000 μ g/ml, as a standard.

1. Bring to room temperature Bradford 1x Dye Reagent and thoroughly mix by inverting the bottle with the Reagent.
2. Bring BSA standards (2000 μ g/ml, 1500 μ g/ml, 1000 μ g/ml, 750 μ g/ml, 500 μ g/ml, 250 μ g/ml, 125 μ g/ml, 0 μ g/ml) stored at -80 °C, to room temperature.

3. Pipet 5 µl in triplicate of each standard to create 0.00, 0.625, 1.25, 2.50, 3.75, 5.00, 7.50 µg/well.
4. Pipet 5 µl unknown protein solution in triplicate (it is necessary to make several dilutions) into wells of 96-well microplate; include a blank/background well containing 5 µl water.
5. Using a 300 µl multichannel pipette quickly dispense 250 µl of Bradford Dye Reagent into wells, including the blank well, using clean tips for each set of wells, cover the plate with the parafilm and mix thoroughly in a microplate reader.
6. Incubate the plate at room temperature for 10 min (at least 5 min and no longer than 1 h). Remove the parafilm and record the absorbance at 595 nm.
7. Determine protein concentration using the microplate reader software which automatically plots standard curve and calculates protein concentration in the unknown samples (Figure 4).

7 Calculating PGLP specific activity.

PGLP specific activity is expressed in µmoles of phosphate liberated per minute per milligram of total protein. An example of calculation: $\frac{\Delta P \times 1000}{5 \times 31 \times \text{protein}}$

ΔP , amount of P in µg produced in 200 µl reaction mix (control values subtracted)

5, time of the reaction in min

31, atom weight of P

Protein, µg of crude protein extract in 200 µl reaction mix

8 Km and Vmax determination for the purified recombinant AtPGLP.

1. Prepare nine 2-PG solutions of different concentrations according to the Table 2, including 0 mM/ml and keep at room temperature.
2. Dilute purified recombinant PGLP to make 0.01 ng/5 μ l (0.002 μ g/ μ l) using 50 mM HEPES pH 7.5, keep on ice.
3. Dilute the phosphate stock 10 times in 50 mM HEPES buffer, pH 7.5, to obtain a diluted stock 5 μ g P/ ml. Pipette the diluted KH_2PO_4 stock solution to produce 0.00, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, and 0.35 μ g P/ml per well in triplicate with final volume of 200 μ l (See Table 1). These samples are used for the standard curve construction.
4. Make a blank/background well: add to a well 200 μ l 50 mM HEPES, pH 7.5.
5. Pipette 187.5 μ l of the reaction buffer into empty wells of 96-well microplate in triplicate for each sample and control using a multichannel pipette.
6. Add 7.5 μ l 2-PG of different concentrations, including 0 mg/ml, into the wells outlined for the activity measurement.
7. Add 5 μ l enzyme solution (0.010 μ g PGLP) into the wells containing 2-PG to initiate reaction and mix. Use the 96-pin replicator to add the enzyme.
8. After 5 min stop reaction by adding 32 μ l of reagent to each well with the reaction mix.
9. For the control in triplicate combine in the following order: 187.5 μ l reaction buffer, 5 μ l enzyme, after 5 min add the reagent to stop any enzymatic reactions followed by addition 7.5

μl of 2-PG. The control adjusts for phosphate that is not produced during the enzymatic reaction.

10. Immediately add 32 μl reagent to each well containing phosphate standards and to the blank well.
11. Cover the plate with parafilm.
12. Record OD at 880 nm after 45 min.
13. Determine phosphate amounts using the spectrometer's software to plot standard curves and calculates amounts of phosphate produced in the wells containing enzymatic reaction mixes. Subtract the values obtained in the corresponding control wells.
14. Calculate specific PGLP activity at each concentration of 2-PG as μmoles phosphate/min/mg protein.
15. Determine K_m and V_{max} using app. (Figure 5).

Notes

1. When larger quantities of purified enzyme are required, it is possible to use 3-4 50 ml separate cultures, or simply one culture of larger volume. Careful attention should be paid to culture aeration. For easier handling, the harvested and frozen cell could be combined for lysis and following sonication in one 50 ml tube.
2. 0.3 M NaCl and 5% glycerol present in Tris buffer help prevent protein precipitation during dialysis.
3. Development of blue color in phosphate assay occurs over time reaching maximum at around 45-60 min.

4. In the event of high phosphate background in phosphate determination assay, all dishes used can be soaked in 3.7% HCl overnight and rinsed thoroughly to remove phosphate contamination.
5. Formation of bubbles during phosphate assay interferes with the linearity of the standard curve. Avoid bubbles by gently adding the reagent to the wells and mixing. If bubbles have formed, spinning the plates for 10-15 min at 1500 g in the swing bucket centrifuge may help.

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14. Quick StartTMBradford Protein Assay. <https://www.biorad.com/webroot/web/pdf/lsr/literature/4110065A.pdf>

Table 1. Dilutions of phosphate stock for standard curve

µg P in 200 µl	P stock (5 µg/ml), µl	50 mM HEPES, pH7.5, µl
0	0 µl	200
0.05	10	190
0.10	20	180
0.15	30	170
0.20	40	160
0.25	50	150
0.30	60	140
0.35	70	130

Table 2. Preparation of 2-PG dilution series for K_M and V_{max} determination.

7.5 μ l 2-PG is added to 200 μ l reaction mix

2-PG in 200 μ l reaction mix, mM	For 1 reaction mix		For 10 reaction mixes	
	2-PG, μ l	H ₂ O, μ l	2-PG, μ l	H ₂ O, μ l
	Need 200 mM 2-PG		Need 200 mM 2-PG	
4	4	3.5	40	35
2	2	5.5	20	55
1	1	6.5	10	65
	Need 20 mM 2-PG		Need 20 mM 2-PG	
0.5	5	2.5	50	25
0.3	3	4.5	30	45
0.1	1	6.5	10	65
	Need 2 mM 2-PG		Need 2 mM 2-PG	
0.05	5	2.5	50	25
0.01	1	6.5	10	65