High throughput glycerate kinase 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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Running Head: High throughput glycerate kinase activity assay

Abstract

We describe an assay for measuring the activity of D-glycerate 3-kinase (GLYK) in a 96-well microplate format with the use of a set of coupling enzymes. The assay is appropriate for use with a crude protein extract prepared from leaf tissue and with the recombinant purified enzyme. The 96-well microplate format reduces the needed amounts of reagents and coupling enzymes, making the assay less expensive, high throughput, and suitable for the determination of kinetic parameters K_m and V_{max}. In addition, we provide a 2-step discontinuous assay modified from past work, making it possible to measure the activity of GLYK at temperatures higher than 45 °C.

Keywords

Photorespiration, D-glycerate 3-kinase, recombinant D-glycerate 3-kinase, 3-phosphoglycerate.

1 Introduction

In chloroplasts, D-glycerate 3-kinase (EC 2.7.1.31) (GLYK) catalyzes the last step in the photorespiratory pathway (PR) to produce 3-phosphoglycerate (3-PGA) from glycerate [1] that enters the C3 cycle thus finishing the recycling of the 2-PG carbon.

Measuring the activity of GLYK involves linking the formation of 3-phosphoglycerate to NADH oxidation with the use of a set of coupling enzymes. The reduced form of NADH absorbs light at 340 nm and its quantity can be directly monitored by absorbance measured continuously with a spectrophotometer. This method can be used in temperatures ranging from 25 °C up to 45 °C. However, if there is a need to measure activity at a temperature higher than 45 ºC the coupling enzymes denature and become inactive making this continuous method impractical.

In this chapter we describe an assay for measuring the activity of GLYK in a 96-well microplate format that uses the same coupling enzymes as routinely used in rubisco activity assays [2, 3] (Figure 1). The assay can be used with a crude protein extract prepared from leaf tissue and preparation of a recombinant purified enzyme. The 96-well microplate format reduces the needed amounts of reagents and coupling enzymes, making the assay less expensive, high throughput, and suitable for the determination of kinetic parameters K_m and V_{max}. In addition, we provide a discontinuous 2-step assay modified from [3] making it possible to measure the activity of GLYK at temperatures higher than 45 ºC.

2 Materials

2.1 Plant material. The protocol described below provides details for measuring GLYK activity using *Nicotiana tabacum* leaves. However, the GLYK activity measurement could be accomplished with any plant tissue of interest that could be homogenized using a set of standard protocols followed by the optimization of the assay. *Nicotiana tabacum* plants were grown in the green house at 30 ˚C (90 ˚F) until tobacco formed 5-7 fully developed leaves [4]. Two fully developed top leaves (located next to each other) were used for tissue sampling. For one sample, three punches 17 mm in diameter (A_{total}=6.81 cm^2, approximately 120 mg) were made, the punches were placed into 2 ml screw cap plastic tubes, frozen immediately in liquid N_2 and stored at -80 °C. In our laboratory the GLYK activity measurement using the outlined protocol was performed in the leaf material from *Rhazya stricta* (Rhazya was grown for approx. 1.5 y; six 8.15 mm in diameter punches, A_{total}=3.14 cm^2, were used to make a homogenate) and birch (at least 1 y old; approximately 120 mg of frozen leaf tissue were used to make a homogenate).

2.2 Common equipment.

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 microplates
8. Pipettes, 2 µl, 20 µl, 300 µl, 1000 µl

9. Multichannel pipette, 300 µl
10. Tips for pipettes 10 µl, 200 µl, 300 µl, 1000 µl
10. 2 ml glass-to-glass homogenizer (Kontes Glass Co., Vineland, NJ, USA)
11. Reservoir for multichannel pipet
12. Polystyrene 96-well microplates
13. Digital Timer
14. Parafilm
15. Oven at 104 °C
16. Volumetric flask 100 ml
17. 15 ml and 50 ml plastic tubes
18. Glass tubes 16 x 125 mm
19. Autoclavable culture tube caps, plastic, or metal
20. Dialysis tubing, or dialysis cassette, or other dialysis system
21. Sterile toothpicks, pointed
22. Incubator with orbital shaking platform for 37 °C and 20 °C
23. HisPur™, or other Ni-NTA Spin columns
24. Sonicator
25. Benchtop liquid nitrogen container
26. Biosafety cabinet
27. Forceps

2.3.1 GLYK activity assay

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 ul 1X Protease Inhibitor Cocktail, EDTA Free. Refer to the Buffer calculation app for amounts of chemicals. Prepare extraction buffer fresh before use.

2. Reaction buffer 50 mM HEPES, pH 7.8, containing 10 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 5 mM creatine phosphate, 0.2 mM NADH [5]. Prepare extraction buffer fresh before use. Refer to the buffer calculation app for the amounts of chemicals.

3. Preparation of coupling enzymes (22.5 U ml^{-1} 3-phosphoglycerate kinase, 12.5 U ml^{-1} creatine phosphokinase, 20 U ml^{-1} glyceraldehyde-3-phosphate dehydrogenase, 20 U mL^{-1} glycerol-3-phosphate dehydrogenase, 56 U ml^{-1} triose-phosphate isomerase):

i. Make 50 mM EPPS-NaOH pH 7.8: 1.62 g EPPS in 90 ml. Adjust pH with 25% NaOH, adjust volume to 100 ml. Filter through 0.22 µm filter using a syringe, store +4 °C.

ii. Combine the following $(NH_4)_2SO_4$ enzyme suspensions in a 15 ml plastic tube:

10000 units 3-phosphoglycerate kinase

8000 units α-glycerophosphate dehydrogenase-triosephosphate isomerase

22600 units triosephosphate isomerase (additional quantities required)

iii. Centrifuge at 10,000 rpm, 20 min, + 4 °C.

iv. Remove supernatant and resuspend the pellet in 6.4 ml 50 mM EPPS-NaOH pH 7.8.

v. Add the remaining enzymes:

10000 units creatine phosphokinase

10000 units glyceraldehyde 3-P dehydrogenase

vi. Dialyze overnight against 50 mM EPPS-NaOH, pH 7.8 at +4 °C. To minimize protein precipitation during dialysis, you may dialyze for 1 h against 2 L buffer, then overnight against fresh 2 L buffer.

vii. Centrifuge at 9,500 g, +4 °C. Retain the supernatant and measure volume. Add glycerol to 20% final concentration. Adjust volume to 8 ml.

viii. Dispense 200 ml aliquots. Store at – 80 °C.

4. D-glyceric acid sodium salt, 500 mM stock: dilute 50 mg in 943 µl H_2O, aliquot by 100 µl, store at -80 °C.

5. Reaction buffer 1 for discontinuous assay: 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 60 mM KCl, 5 mM ATP.

6. Reaction buffer 2 for discontinuous assay: 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 60 mM KCl, 15 mM NADH, 2 mM ATP, 10 mM creatine phosphate.

2.3.2 Expression and purification of recombinant AtGLYK

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

2. Luria-Bertani (LB) medium, liquid.

3. LB agar medium (15 g/L) in Petri dishes containing 50 ug/ml kanamycin.

4. 80% glycerol, sterile.

5. 1 M IPTG (isopropyl ß-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 ºC.

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 $CaCl_2$, 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.

2.3.3 Protein concentration determination

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 ug/ml, 1500 ug/ml, 1000 ug/ml,750 ug/ml, 500 ug/ml, 250 ug/ml, 125 ug/ml), store at –80 °C.

3 Preparation of crude protein extract.

1. For one sample, collect 3 *Nicotiana tabacum* punches (each 17 mm in diameter; A_{total}=6.81 cm^2, total weight approx. 120 mg) using fully developed top leaves located next to each other. Using clean forceps quickly place the punches into a 2 ml screw cap plastic tubes, froze immediately in liquid N_2 and store at -80 °C until analysis. Do not allow to thaw until

analysis. Using the described procedure, collect and freeze as many samples as required for the analyses.

2. Homogenize the frozen 3 leaf punches manually in 1 ml of the extraction buffer with the use of a 2 ml glass-to-glass homogenizer (Kontes Glass Co., Vineland, NJ, USA) kept on ice, about 3-5 min.

3. Transfer the homogenate into a 1.5 ml plastic tube kept on ice and clarify by centrifugation for 10 min at 13,500 g, +4 °C. The supernatant represents the crude protein extract and is used for the enzyme assay immediately. (Note 1).

4 Expression and purification of a recombinant *Arabidopsis thaliana* GLYK (AtGLYK).

Expression of recombinant N-terminal 6xHis tagged AtGLYK containing no targeting peptide was performed using NEB T7 express *E. coli* cell line transformed with a plasmid pET28a AtGLYK (DOE JGI Statement). The plasmid is available upon the request. Under described conditions at least a part of the recombinant protein is soluble according to the SDS-PAGE data. The outlined procedure results in 90% or higher purity of the isolated recombinant protein with the yield around 6 -7 mg/ml.

4.1 Expression of recombinant AtGLYK

1. Inoculate 3 ml of LB containing kanamycin (add 3 µl kanamycin from 50 mg/ml stock) in a glass tube with a single colony from agar plate (LB+Kan) using a sterile toothpick or sterile 10 µl tip; perform these procedures under a sterile biosafety hood. Cover the tube with a culture tube cap. Grow the culture at 37 °C overnight, 225 rpm.

2. The next day, inoculate 50 ml of pre-warmed LB media (kanamycin 50 µg/ml) in 250 ml flask with 0.5 ml of the overnight culture and grow at 37 °C with vigorous shaking until an OD_{600} of 0.6-0.8 is reached (~2.5 h). Check OD_{600} frequently.
3. Place 1 ml sample into 1.5 ml plastic tube immediately before induction, this sample is non-induced culture. Pellet cells 10 min, 3500 g, at +4 °C, discard supernatant, freeze the cell pellet on dry ice, store at -20 °C until analysis by SDS-PAGE.
4. Pre-cool culture to 20 °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 culture overnight at 20 °C; the optical density reaches ~ 2. Collect a second 1 ml sample. This sample is the induced culture; pellet cells (10 min, 3500 g, +4 °C), freeze and store the sample at –20 °C for SDS-PAGE analysis.
6. Harvest the cells from 50 ml culture by centrifugation at 4200 *g* for 20 min, +4 °C, in 50 ml plastic tube.
7. Completely remove supernatant and freeze the cells in dry ice or liquid nitrogen. Use the frozen pelleted cells for purification immediately or store the cell pellet at –80 °C until purification procedure.

4.2 Purification of recombinant AtGLYK

Binding, washing, and elution are basically performed according to the Qiagen Ni-NTA Spin Kit Handbook [6] with modifications outlined below. Perform all procedure at +4 °C.

1. Prepare 50 mM Tris-HCl, 300 mM NaCl, pH 8.0. Dissolve 6.06 g Tris and 17.53 g NaCl per 1 L water. Adjust pH to 8.0 with conc. HCl. Keep at +4 °C. (Note 2).

2. Thaw the cell pellet for approx.15 min on ice and re-suspend the cells in lysis buffer: add 1.5 ml to the cells obtained from 50 ml induced culture. (See Note 3).

3. Incubate 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 (e.g., Branson Sonicator) at high frequency, with 10 sec intervals for cooling.

5. Centrifuge 20 min, 19800 g, +4 °C (centrifugation could be performed in 1.5-2 ml plastic tubes).

6. Collect the supernatant (this is the crude soluble protein extract) into 15 ml conical tube, keep on ice. Take 20 µl supernatant and freeze at -20 °C for SDS-PAGE analysis.

7. 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.

8. Apply 600 µl supernatant onto an equilibrated HisPur Ni-NTA column placed into a 2-ml collection tube. Centrifuge the column with the tube at 50 g, 2 min, +4 °C (See Note 2). Discard the flow-through but collect 20 µl, store at -20 °C until SDS-PAGE analysis.

9. Repeat applying 600 µl supernatant onto the column followed by centrifugation at 50 g, 2 min, +4 °C, until all supernatant has been loaded.

10. Wash 2-3 times by applying 600 µl NP1-20 followed by centrifugation at 890 g for 2 min, +4 °C. Discard washes, but collect 20 µl, store at -20 °C until SDS-PAGE analysis.

11. Place the column into a clean 1.5-ml collection tube. Elute the protein twice with 300 µl of NP1-500 followed by centrifugation at 890 g for 2 min, +4 °C (See Note 3).

12. Combined eluates (2x300 µl) (You may keep eluates separately) 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 (See Note 4).
13. The next day, if protein precipitation is present, remove the precipitated protein by centrifugation at 14,000 rpm, +4 °C for 10 min.
14. Measure protein concentration in the supernatant using NanoDrop and/or Bradford protocol for microplate.
15. Add 80% sterile glycerol to final 10%, aliquot, store at -80 °C.
16. Using SDS-PAGE (not provided in this Chapter) analyze the non-induced and induced cultures, crude soluble protein extract, flow-through, washes, and eluates.

5 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 µl microplate assay format with using BSA in the linear range 125–1,000 µg/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 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 0 µg/ml) stored at -80 °C, to room temperature.

3. Pipet 5 µl in triplicate of each standard to the wells 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 in water) into wells of 96-well microplate; include a blank/background well containing 5 ul 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).

6 Pathlength determination method for 96-well microplate. To accurately estimate the activity of GLYK using a 96-well format it is necessary to determine pathlength of the measured absorbance as this depends on the volume and the composition of the reaction mix, as a microplate reader measures the absorbance from the top or bottom of the plate. Here we provide a method to determine the pathlength in a standard flat bottom 96-well microplate with a volume of reaction mix of 200 microliters per well using Beer's law.

1. Make fresh 0.15 mM solution of NADH in a 50 mM HEPES buffer, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, and determine optical density at 340 nm in a quartz cuvette with pathlength l = 1 cm, 25 °C. Measure concentration of NADH using Beer's Law: Abs = Ɛ x C x l with extinction coefficient for NADH equal 6.22 x 10^3 M^{-1} x cm^{-1}, and l equal 1 cm.

2. Dispense 196 µl NADH solution into a well of a polystyrene plate, add 4 µl of extraction buffer, measure optical density at 340 nm and, applying Beer's Law: Abs = Ɛ x C x l, determine l using extinction coefficient for NADH equal 6.22 x 10^3 M^{-1} x cm^{-1} and NADH concentration measured earlier. The pathlength was equal 0.56 cm at 25 °C in our experiments.

7 Glycerate kinase activity assay.

The activity of GLYK is measured by linking the formation of the product of the GLYK reaction 3-phosphoglycerate to NADH oxidation using a set of coupling enzymes analogous to the set of coupling enzymes, excluding carbonic anhydrase, used for measuring the rubisco activity (Figure 1).

7.1 GLYK activity assay to perform at 25 ºC – 45 ºC.

1. Dispense 192 µl reaction buffer containing 50 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 5 mM creatine phosphate, 0.2 mM NADH into wells of 96-well microplate in triplicate according to the number of samples.
2. To the wells add 4 µl crude protein extract or 4 µl purified GLYK (50 ng), and 4 µl coupling enzymes.
3. Take measurements at 340 nm every 10-15 sec for 2 min without added substrate to establish the baseline.
4. Initiate the reaction by adding 2 µl 500 mM D-glycerate (5 mM final) with the use of a 96-well microplate replicator.

5. Monitor the decrease in optical density at 340 nm for 5-10 min. The initial linear rate of reaction is used to express the specific activity as umoles NADH utilized per minute per milligram of total protein. Based on the coupling enzyme set used, formation of every mole of 3-phosphoglycerate corresponds to 2 moles of oxidized NADH (Figure 1).

7.2 The discontinuous two-step GLYK activity assay to perform at a temperature higher than 45 ºC.

Protocol

i. The first step is performed in a 2 ml plastic tube at a temperature higher than 45 °C.

1. Dispense 388 µl reaction buffer 1 into 2 ml plastic tubes, warm up to desired temperature in a heating block.
2. Add 4 µl 500 mM D-glyceric acid, mix.
3. Initiate the reaction by adding 8 µl containing 100-200 ng recombinant GLYK, mix and close the tube. Using a timer keep the tube at the desired temperature for 1 min.
4. Stop the reaction by transferring the tube into a heating block at 95 °C for 5 min, then bring to room temperature.

ii. The second step is performed in a 96-well microplate at 30 °C:

1. Dispense 96 µl reaction buffer 2 per well to a pre-warmed plate. The final volume of the reaction mix in the first step allows to make 3 replicates.
2. Add 100 µl per well of the reaction mix obtained at the first step.
3. Observe the initial optical density at 340 nm after 2 min using the microplate reader.

4. Add 4 µl of coupling enzymes per well, monitor optical density at 340 nm after 2 min using the microplate reader. Add coupling enzymes using a multichannel pipette or a 96-pin microplate replicator if many samples are being analyzed.
5. For activity calculation use the difference between the initial values of optical density and optical density obtained after addition of coupling enzymes; subtract the background optical density values contributed by coupling enzymes only.

8 Km and Vmax determination for the purified recombinant AtGLYK.

1. Prepare D-glycerate solutions of 10 concentrations according to table 1, including 0 mM/ml, keep at room temperature.
2. Make reaction buffer 50 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 5 mM creatine phosphate, 0.2 mM NADH.
3. Dispense 192 µl reaction buffer into wells of a 96-well microplate in triplicate according to the number of samples.
4. To the wells add 4 µl purified GLYK containing 50 ng enzyme and 4 µl coupling enzymes.
5. Take measurements at 340 nm every 10-15 sec for 2 min without added substrate to establish the baseline.
6. Initiate the reaction by addition of 2 µl D-glycerate of different concentrations including 0 mM with the use of a 96-well microplate replicator.
7. Monitor the decrease in optical density at 340 nm for 5 min. Subtract the baseline. The initial linear rate of reaction is used to express the specific activity as µmoles NADH utilized per minute per milligram of total protein. Note, based on the coupling enzyme set used, formation of every mole of 3-phosphoglycerate corresponds to 2 moles of oxidized NADH.

8. Calculate specific GLYK activity at different concentrations of glycerate.

9. Determine K_m and V_{max} using the R Shiney App available here: (https://github.com/PerennialDr/MichaelisMenten_fit) or any other compatible fitting method.

Notes

1. The crude protein extract prepared using tobacco leaves and stored at +4 °C overnight loses approximately 10% of the activity determined immediately after the extract was made.
2. For dialysis prepare 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, fresh or a day before and store at +4 °C.
3. 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 aeration during culture growth. For easier handling, the harvested and frozen cell could be combined for lysis and following sonication in one 50 ml tube.
4. Low speed during centrifugation provides more time for the enzyme to bind the Ni-matrix.
5. The elution of recombinant GLYK could be performed by 300 mM imidazole.
6. 300 mM NaCl and 5% glycerol in the dialysis buffer help to prevent protein precipitation.

Table 1: D- Glycerate dilutions for Km and Vmax determination.

Glycerate in 200 ul reaction mix, mM	For 1 reaction mix		For 20 reaction mixes	
	Needed 500 mM glycerate, µl	Needed H_2O, µl	Needed 500 mM glycerate, µl	Needed H_2O, µl
5	2	0	40	0
2	0.8	1.2	16	24
1	0.4	1.6	8	32
0.5	0.2	1.8	4	36
	Needed 50 mM glycerate, µl	Needed H_2O, ul		
0.2	0.8	1.2	16	24
0.1	0.4	1.6	8	32

0.05	0.2	1.8	4	36
	Needed 5 mM glycerate, µl	**Needed H_2O, µl**		
0.02	0.8	1.2	16	24
0.01	0.4	1.6	8	32
0	0	2	0	40

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