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Measurement of Glucose-6-phosphate Dehydrogenase Activity in Bacterial Cell-free Extracts

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[Abstract] Glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) is the first enzyme of the oxidative pentose phosphate cycle and catalyses the conversion of glucose-6-phosphate (G6P) to 6-phosphoglucono-δ-lactone and transfers one electron to $NADP^+$ producing one NADPH. Conversion of G6P to 6-phosphoglucono-δ-lactone is proportional to the production of NADPH. The increase in NADPH concentration results in an increase in absorbance at 340 nm. To assay G6PDH activity, therefore, production of NADPH is determined by measuring increase in absorbance at 340 nm spectrophotometrically. This increase rate is then converted to unit of activity and specific activity of G6PDH. In this procedure, a generalized method is given for bacterial G6PDH assays emphasizing on a cyanobacterium *Synechocystis* sp. PCC6803 (Schaeffer and Stanier, 1978; Karakaya *et al.*, 2008, 2012) and a heterotrophic bacterium *E.coli* (Hylemon and Phibbs, 1972; Barnel *et al.*, 1990).

Materials and Reagents

1. 1.5 ml Eppendorf tubes (Eppendorf, catalog number: 022363204)
2. Micropipette tips (200 µl) (Sigma-Aldrich, catalog number: CLS4866)
3. Micropipette tips (1,000 µl) (Sigma-Aldrich, catalog number: CLS4868)
4. Glass beads (unwashed) (212-300 µm) (Sigma-Aldrich, catalog number: G9143)
5. 1.5 ml polystyrene spectrophotometer cuvettes with 10 mm path length (Sigma-Aldrich, catalog number: C5416)
6. Parafilm (Sigma-Aldrich, catalog number: P7793)
7. Bacterial cells

   Note: Amount of the cell depends on how many assays will be carried out. Supernatant yielded from a cell pellet of 50 ml well-grown cyanobacterial culture (OD_{750} ≥ 1.0) and 10 ml overnight grown Escherichia coli will be sufficient for about 20 assays depending on what volume is used for each assay.
8. Trizma® base (Sigma-Aldrich, catalog number: T1503)
9. β-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
10. Glucose-6-phosphate disodium salt (G6P) (Sigma-Aldrich, catalog number: G7250)
11. Potassium phosphate dibasic trihydrate ($K_2HPO_4{\cdot}3H_2O$) (Sigma-Aldrich, catalog number: P5504)
12. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P0662)

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13. Magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, catalog number: M7506)
14. Hydrochloric acid (HCl) (36.5-38.0%) (Sigma-Aldrich, catalog number: H1758)
15. Maleic acid (Sigma-Aldrich, catalog number: M0375)
16. β-nicotinamide adenine dinucleotide phosphate hydrate ($NADP^+$) (Sigma-Aldrich, catalog number: N5755)
17. Paraffin
18. 50 mM Tris-maleate solution (see Recipes)
19. Extraction buffers
    a. Tris-maleate buffer, pH 6.8 for *Synechocystis* sp. PCC6803 (see Recipes)
    b. Potassium phosphate buffer, pH 6.8 for *E.coli* (see Recipes)
20. Assay buffers
    a. Tris-maleate buffer, pH 7.4 for *Synechocystis* sp. PCC6803 (see Recipes)
    b. Tris-HCl buffer, pH 8.0 for *E.coli* (see Recipes)
21. 500 mM Tris-HCl stock solution (pH 8.0) (see Recipes)
22. 500 mM G6P solution (see Recipes)
23. 100 mM $NADP^+$ solution (see Recipes)

Equipment

1. Micropipettes (20-200 µl capacity) (Nichiryo, catalog number: 00-NPX2-200)
2. Micropipettes (100-1,000 µl capacity) (Nichiryo, catalog number: 00-NPX2-1000)
3. Microcentrifuge, 1.5 ml Eppendorf tube rotor and at least 10,000 *x g* force (Sigma-Aldrich, Hettich®, model: MIKRO120)
4. FastPrep FP120 (BioSurplus, Thermo-Savant, model: FP120)
5. Light microscope
6. Vortex mixer (Bibby Scientific, Stuart, model: SA8)
7. Balance (Precision Weighing Balances, Salter Brecknell, model: ESA-150)
8. pH meter (Hanna Instruments, model: HI5221)
9. Vis-Spectrophotometer with 1.5 ml cuvette holder (Shimadzu Scientific Instruments, model: UV-1800)

Procedure

A. Extraction of G6PDH from bacterial cells

Note: Bacterial cells and cell-free extracts must be kept on ice or at 4 °C throughout all steps of extraction.

1. Resuspend the cell pellets in 500 µl extraction buffer (Tris-maleate buffer pH 6.8 for cyanobacterial cells or potassium phosphate buffer pH 6.8 for *E.coli* cells). The extraction buffers keep G6PDH enzyme in an active state.

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Note: G6PDH of the photosynthetic prokaryotes like cyanobacteria is a redox modulated oligomeric enzyme. In case, redox modulation properties are planned to be tested, β-mercaptoethanol must be omitted from the buffers.

2. To wash glass beads, add 500 µg glass beads and 1 ml extraction buffer to two separate Eppendorf tubes, mix with a whirly mixer well, centrifuge at 10,000 *x g* for 1 min, and discard the supernatant.
3. Add 500 µl cell suspension to each glass beads containing tubes and mix the tube to resuspend the glass beads.
4. Put the tubes in fast prep FP120 and run the device twice at 5.5 m/sec for 40 sec.
   Note: This step may be done by mixing the tubes vigorously on a whirly mixer for 1-2 min until most of the cells are disrupted. This may be confirmed by examining a small amount of the crude extract for unbroken cells under a light microscope.
5. Centrifuge the extract at 10,000 *x g* for 10 min.
6. Transfer the supernatant to a new Eppendorf tube and keep at 4 °C. Use this cell-free extract as enzyme solution.
   Note: It is better to use the extract immediately. However, the extract may be stored at 4 °C for a week albeit some degree of activity loss.

B. Assay of G6PDH activity in cell-free extract

1. Set the wavelength of spectrophotometer to 340 nm. Select absorbance function.
2. Prepare a blank and a test mixture as follows (Table1).

Table 1. Regent contents of test and blank mixtures

	Test	Blank
Assay buffer	770 µl	800 µl
$NADP^+$ (50 mM)	30 µl (1.5 mM)	-
Enzyme solution	50 µl	50 µl
G6P (100 mM)	150 µl (15 mM)	150 µl (15 mM)

3. Add assay buffer, $NADP^+$ and enzyme solutions first to a 1 ml spectrophotometer cuvette, then, start the enzyme reaction by adding G6P and mix the content by flipping the paraffin sealed cuvette.
   Note: Assay buffer is Tris-maleate (pH 7.4) for cyanobacteria and Tris-HCl (pH 8.0) for E.coli. A blank reaction mixture is needed to check any endogenous $NADP^+$ reduction in enzyme solution. A minimum amount of 200 mg protein per assay is necessary albeit it may vary depending on factors such as growth phase of the culture used.
4. Place the cuvette in the cell holder of the spectrophotometer, close the lid and follow the absorbance for 3-5 min at room temperature by recording absorbance value in 15-30 sec intervals. Temperature of the reaction mixture should be kept at a certain point. Some enzymes

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need specific temperature like 37 °C. In this case reaction cuvette must be treated with a thermostatic apparatus integrated to the spectrophotometer. However, some enzymes work well at room temperature.

5. Estimate ΔA_{340} (rate) dividing the total absorbance change to the total assay time in min. For example, if the absorbance change in a reaction mixture at 340 nm is 0.450 after 3 min, the rate is 0.150.
   Note: Some spectrophotometers have rate measurement function. Using such a device, the rate may be determined directly.
6. Repeat steps B4 to B6 for the blank tube to test whether any detectable endogenous $NADP^+$ is present in the enzyme solution. If so, subtract this value from the test value to find net ΔA_{340} value.

C. Estimation of unit of activity and specific activity of G6PDH

One of the standard expression ways of enzyme activity is the unit of activity. Unit of G6PDH activity is defined here as the formation of 1 µmol NADPH in one min. $NADP^+$ reduction (G6PDH activity) determined as rate (ΔA_{340}) is needed to be converted to concentration in µmol. Once the unit of activity is determined, it is often converted to specific activity. Specific activity is expressed as units per mg protein.

1. Estimate G6PDH activity in units by using the formula as follows:

$$Units\ of\ Activity\ (uMol\ min^{-1}\ ml^{-1}) = \frac{(\text{Rate})\text{x}\ (\text{Dilution factor of enzyme solution})}{\text{Extinction coefficient for NADPH}\ (6{,}22\ \text{mM}^{-1}\text{cm}^{-1})}$$

   In this protocol the dilution factor is 20 (50 µl extract in 1,000 µl test or blank mixture). For example, if a rate of 0.85 is yielded in the test assay and 0.05 in the blank assay, the net rate will be 0.8. Then the units of activity will be estimated as 0.8 x 20/6.22 = 2.572 µmol min^{-1} ml^{-1}.
2. To estimate specific activity, the protein amount in the extract must be determined by a standard method such as Bradford assay. Specific activity is then estimated as the amount of units per mg protein by using the formula below:

$$Specific\ Activity\ (units/mg\ protein) = \frac{Amount\ of\ activity\ units}{Protein\ concentration\ of\ enzyme\ solution\ (mg\ ml^{-1})}$$

   For example, if the protein content of the extract is 0.5 mg ml^{-1}, the specific activity will be 2.572/0.5 = 5.44 units/mg protein.

Notes

1. One important point is to keep the enzyme in the active state in the supernatant especially by protecting it from oxidation damage. To avoid oxidation damage and keep the enzyme in the active state, the reducing agent β-mercaptoethanol may be added to the supernatant. However,

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this agent also reduces disulphide bonds in the enzyme and should be omitted when studying the redox properties of the enzyme.

2. It is known that higher amounts of G6PDH, G6P and NADP positively affect the enzyme's activity. If the effects of some agents on G6PDH activity are studied, minimal amounts of enzyme solution and substrates should be used. It is difficult to define standard minimal concentrations for enzyme and substrates since several factors may affect these values. Therefore, the researchers must determine minimal concentrations themselves.

Recipes

1. 50 mM Tris-maleate solution
   Dissolve 6.05 g Trizma-base in 800 ml water and adjust pH to 6.8 (for extraction buffer) or 7.4 (for assay buffer) by adding maleic acid.
   Keep the solution at room temperature for a few months.
2. Tris-maleate extraction buffer
   50 mM Tris-maleate (pH 6.8)
   0.1% β-mercaptoethanol
   10 mM G6P
   Mix the reagents just before use.
3. Tris-maleate assay buffer
   50 mM Tris-maleate (pH 7.4)
   0.1% β-mercaptoethanol
   10 mM $MgSO_4$
4. Potassium phosphate extraction buffer (pH 6.8) (Barnell *et al*., 1990)
   50 mM K_2HPO_4
   50 mM KH_2PO_4
   Titrate against each other to adjust pH to 6.8.
5. 500 mM Tris-HCl stock solution pH 8.0
   Dissolve 60.57 g Trizma base in 800 ml water and adjust pH to 8.0 with 2 N HCl solution.
   Keep the solution at room temperature for a few months.
6. Tris-HCl assay buffer for *E.coli* (Hylemon and Phibbs,1972)
   50 mM Tris-HCl (pH 8.0)
   0.1% β-mercaptoethanol
   10 mM $MgSO_4$
7. 500 mM G6P solution
   Prepare 500 mM G6P solution (152.05 mg G6P in 1 ml ultrapure water), separate in aliquots and store at -20 °C for a few months.

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8. 100 mM $NADP^+$ solution
   Prepare 100 mM $NADP^+$ solution (74.34 mg $NADP^+$ in 1 ml ultrapure water), separate in aliquots and store at -20 °C for a few months.

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