

Separation of Plant 6-Phosphogluconate Dehydrogenase (6PGDH) Isoforms by Non-denaturing Gel Electrophoresis

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[Abstract] 6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) catalyzes the third and irreversible reaction of the pentose phosphate pathway (PPP). It carries out the oxidative decarboxylation of the 6-phosphogluconate to yield ribulose-5-phosphate, carbon dioxide and NADPH. In higher plants, 6PGDH has several subcellular localizations including cytosol, chloroplast, mitochondria and peroxisomes (Corpas *et al.*, 1998; Krepinsky *et al.*, 2001; Mateos *et al.*, 2009; Fernández-Fernández and Corpas, 2016; Hölscher *et al.*, 2016). Using *Arabidopsis thaliana* as plant model and sweet pepper (*Capsicum annuum* L.) fruits as a plant with agronomical interest, this protocol illustrates how to prepare the plant extracts for the separation of the potential 6PGDH isoforms by electrophoresis on 6% polyacrylamide non-denaturing gels. Thus, this method allows detecting three 6PGDH isoforms in *Arabidopsis* seedlings and two 6PGDH isoforms in sweet pepper fruits.

Keywords: NADPH, Non-denaturing polyacrylamide gel electrophoresis, Pentose phosphate pathway, 6-Phosphogluconate dehydrogenase

[Background] Non-denaturing gel electrophoresis is a powerful technique that allows separating native proteins. Their mobility depends of protein size, shape and net charge. In these analytical conditions, the protein preserves its activity and in combination with a specific staining method, it allows to separate the presence of potential isoforms. This approach has been widely used in the case of the family of superoxide dismutases. However, to our knowledge, there are not many papers that analyze the presence of different isoforms of 6PGDH activity in plant tissues (Corpas *et al.*, 1998; Mateos *et al.*, 2009). This straight method may be very useful for researchers working with plant 6PGDHs.

Materials and Reagents

1. 10-cm-diameter Petri dishes
2. *Arabidopsis thaliana* ecotype Columbia seeds (originally obtained from NASC, Nottingham Arabidopsis Stock Center)
3. Sweet green pepper fruits were provided by Syngenta Seeds S.A. (El Ejido, Spain)
4. 70% (v/v) ethanol
5. 0.1% (w/v) SDS

6. Commercial Bleach
7. Murashige and Skoog medium (Sigma-Aldrich, catalog number: M5524)
8. Phyto-agar (Duchefa Biochemie, catalog number: P1001)
9. Sucrose (Sigma-Aldrich, catalog number: 84097)
10. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 5000006)
11. Bovine serum albumin (BSA) Fraction V (Roche Diagnostics, catalog number: 10735078001)
12. Tris (AMRESCO, catalog number: 0497)
13. Ethylenediaminetetraacetic acid, disodium salt, dihydrate (Na₂-EDTA) (Sigma-Aldrich, catalog number: E5134)
14. Triton X-100 (AMRESCO, catalog number: 0694)
15. Glycerol (AMRESCO, catalog number: E520)
16. Dithiothreitol (DTT) (Roche Diagnostics, catalog number: 10708984001)
17. 30% acrylamide/Bis solution, 19:1 (Bio-Rad Laboratories, catalog number: 1610154)
18. 4x gel buffer
19. Glycine (AMRESCO, catalog number: 0167)
20. Ammonium persulfate
21. TEMED
22. β-Nicotinamide adenine dinucleotide phosphate, sodium salt, hydrate (NADP) (Sigma-Aldrich, catalog number: N0505)
23. Magnesium chloride, hexahydrate (MgCl₂·7H₂O) (EMD Millipore, catalog number: 442615)
24. Nitroblue tetrazolium (NBT) (AMRESCO, catalog number: 0329-1G)
25. Phenazine methosulfate (PMS) (Sigma-Aldrich, catalog number: P9625)
26. 6-Phosphogluconic acid, trisodium salt (6PG) (Sigma-Aldrich, catalog number: P7877)
27. Glacial acetic acid (Fisher Scientific)
28. Grinding buffer (see Recipes)
29. Non-denaturing polyacrylamide gel electrophoresis (PAGE) on 6% acrylamide gel (see Recipes)
30. Staining solution (see Recipes)
31. Stop solution (see Recipes)

Equipment

1. Plant Growth cabinet (Panasonic Biomedical, model: MLR-352-PE)
2. Porcelain mortar and pestle
3. Refrigerated centrifuge
4. Vertical Slab gels Electrophoresis System (Bio-Rad Laboratories, model: Mini-PROTEAN® Electrophoresis Cell)

Software

1. ImageJ program (<https://imagej.nih.gov/ij/>)

Procedure

A. Plant extracts

1. Surface-sterilize *Arabidopsis thaliana* ecotype Columbia seeds for 5 min in 70% (v/v) ethanol containing 0.1% (w/v) SDS at room temperature and with a slight shaking. Then, place for 20 min in sterile water containing 20% (w/v) commercial Bleach and 0.1% (w/v) SDS and then wash four times in sterile water.
2. Grow seeds on Petri plates over commercial Murashige and Skoog medium (Sigma-Aldrich) with a pH of 5.5, containing 1% (w/v) sucrose and 0.8% (w/v) phyto-agar (Corpas and Barroso, 2014). Then, keep the seedlings for 14 days with a photoperiod of 16 h (light intensity of 100 $\mu\text{E m}^{-2} \text{sec}^{-1}$) and 8 h dark, with a temperature of 22 °C and 18 °C, respectively.
3. Sweet green pepper fruits were provided by Syngenta Seeds S.A. (El Ejido, Spain) from plants grown in experimental greenhouses.
4. Homogenize plant samples in a mortar and pestle with grinding buffer (see Recipes) in a ratio 1:3 (w/v) for *Arabidopsis* and ratio 1:1 for pepper fruits. Perform these operations at 0-4 °C (on ice).
5. Centrifuge extracts at 27,000 $\times g$ at 4 °C for 20 min.
6. Use the supernatants for the enzymatic assays. Determine protein concentration with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

B. 6PGDH activity staining

1. Separate plant proteins by non-denaturing polyacrylamide gel electrophoresis (PAGE) on 6% acrylamide gels at 4-7 °C. Usually, it takes around 2 h and 30 min.
2. After electrophoresis, incubate gels in staining solution (see Recipes) at room temperature (RT). Keep in the dark with shaking until blue-purple bands appear over a colorless background. It takes around 15 min (Figure 1).
3. Remove the staining solution and add stop solution (see Recipes) to cover gels.

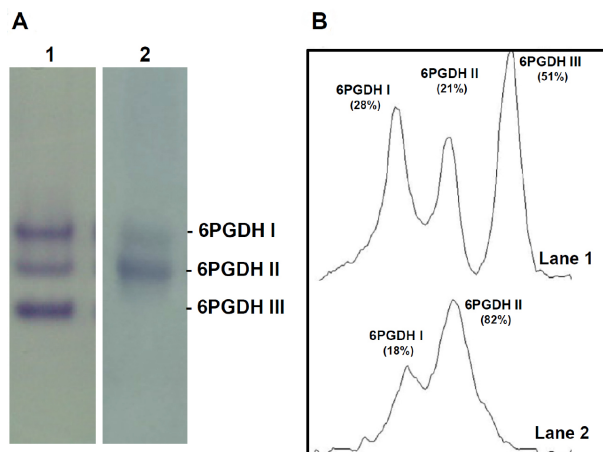


Figure 1. Analysis of 6-phosphogluconate dehydrogenase (6PGDH) isoforms by non-denaturing gel electrophoresis. A. 6PGDH isoforms activity were separated by native PAGE (6%) and identified by activity staining. Lane 1, *Arabidopsis thaliana* 14-day-old seedlings. Lane 2, sweet green pepper fruits. B. Densitometric scans of 6PGDH isoforms and its relative quantification (%) made by the ImageJ program.

Note: It is recommended to use fresh vegetable extracts because they provide better defined bands of activity.

Data analysis

Quantification of the activity of each 6PGDH isoform can be done by densitometric analysis (Figure 1B), for example, using the ImageJ program.

Recipes

1. Grinding buffer
 - 50 mM Tris-HCl, pH 7.8 containing 0.1 mM EDTA
 - 0.2% (v/v) Triton X-100
 - 10% (v/v) glycerol
 - 2 mM DTT
2. Non-denaturing polyacrylamide gel electrophoresis (PAGE) on 6% acrylamide gel
 - 30% acrylamide/Bis solution, 19:1
 - 4x gel buffer (1.5 M Tris-HCl, pH 8.9)
 - Electrode buffer (38 mM glycine plus 5 mM Tris, pH 8.2)
 - 10% (w/v) ammonium persulfate (AP)
 - TEMED

The follow table shows the needed volume of each reagent to prepare two 1.5 mm thick gels to be used in a Bio-Rad Mini-PROTEAN Electrophoresis System

Reagent	6%
30% acrylamide/Bis solution	5.40 ml
Gel buffer	6.80 ml
MiliQ water	13.45 ml
Vacuum (10 min)	
*7% (w/v) ammonium persulfate (AP)	1.45 ml
*TEMED	0.10 ml

**Note: Added right before each use. The mix of the reagents should be done at 4 °C (on ice).*

The standard running conditions are 15 mA per gel for 30 min and then increase to 25 mA per gel for approximately 90 min. The electrophoresis should be run in a cold room (5-7 °C)

3. Staining solution

50 mM Tris-HCl, pH 7.6 containing 0.8 mM NADP

5 mM EDTA

2 mM MgCl₂

0.24 mM NBT

65 mM PMS

10 mM 6PG

Note: Freshly prepared before use and protected from the light.

4. Stop solution

7% (v/v) acetic acid

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