Extraction and Measurement the Activities of Cytosolic Phosphoenolpyruvate Carboxykinase (PEPCK) and Plastidic NADP-dependent Malic Enzyme (ME) on Tomato (*Solanum lycopersicum*)

Sonia Osorio[1*], José G. Vallarino[1], Marek Szecowka[2], Shai Ufaz[3], Vered Tzin[3], Ruthie Angelovici[3], Gad Galili[3] and Alisdair R. Fernie[4]

[1]Department of Molecular Biology and Biochemistry, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" – University of Malaga- Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Malaga, Spain; [2]Central Metabolism Research Group, Max-Planck-Institut für Molekulare Planzenphysiologie, Potsdam-Golm, Germany; [3]Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel

*For correspondence: sosorio@uma.es

[Abstract] A recent study demonstrated that cytosolic phosphoenolpyruvate carboxykinase (PEPCK) and NADP-malic enzyme (NADP-ME) have an important role in malate metabolism during fruit ripening (Osorio *et al.*, 2013). PEPCK catalyze the ATP-dependent decarboxylation of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and NADP-ME, the reversible conversion of malate and pyruvate. Here, we present the detailed protocols to measure PEPCK activity in carboxylation direction by following oxidation of NADH and to measure NADP-ME activity based upon the reduction of $NADP^+$.

Materials and Reagents

A. PEPCK activity

1. Un-harvested plant tissues (how to freeze the tissue is explained in the procedure section)
2. Liquid N_2
3. Ice
4. Bicine (Sigma-Aldrich, catalog number: B8660)
5. KOH
6. EDTA (Sigma-Aldrich, catalog number: EDS-100G)
7. Poly (ethylene glycol) - 4000 (Sigma-Aldrich, catalog number: 81240)
8. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 43815)
9. β-nicotinamide adenine dinucleotide reduced form (NADH) (Roche Diagnostics, catalog number: 10128015001)
10. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (Sigma-Aldrich, catalog number: H3375)

11. KCl
12. $MnCl_2$
13. Phosphoelnolpyryvate (PEP) (Bio Vectra, catalog number: 2552)
14. Adenosine 5′-diphosphate sodium salt (ADP) (Sigma-Aldrich, catalog number: A2754)
15. $KHCO_3$
16. L-Malate dehydrogenase (Roche Diagnostics, catalog number: 10127248001)
17. Bradford stock solution (Bio-Rad Laboratories, catalog number, 500-0006)
18. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2058)
19. Extraction buffer 1 (see Recipes)
20. Extraction buffer 2 (see Recipes)
21. Buffer 3 (see Recipes)
22. PEPCK assay mix 1 (see Recipes)

B. NADP-ME activity
1. Un-harvested plant tissues (how to freeze the tissue is explained in the procedure section)
2. Liquid N_2
3. Ice
4. Tris-Base (United State Biological, catalog number: T8600)
5. $MnCl_2$
6. EDTA (Sigma-Aldrich, catalog number: EDS-100G)
7. Glycerol (Sigma-Aldrich, catalog number: G5516)
8. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
9. β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Roche Diagnostics, catalog number: 10128058001)
10. L-Malate (Sigma-Aldrich, catalog number: M1000)
11. Bradford stock solution (Bio-Rad Laboratories, catalog number, 500-0006)
12. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2058)
13. Extraction buffer 4 (see Recipes)
14. NADP-ME assay mix 1 (see Recipes)

Equipment

1. Small mortar and pestle
2. 2 ml and 1.5 ml microfuge tubes
3. Pipettes
4. Balance
5. 2 ml centrifuge (Hettich Mikro 22R)

6. 96 well polystyrene microplate (flat bottom) (Corning, catalog number: 3300)
7. A computer supported microplate spectrophotometer for kinetic (time-course) measurement mode (Elisa microplate-spetrophotometer) (BioTek Instruments, model: EL808)

Procedure

A. Extraction for measuring PEPCK activity

This protocol applies to extraction of PEPCKs from plant tissues.

1. Collect the sample and freeze immediately in liquid N_2. Store at -80 °C until use.
2. Add small amount of liquid N_2 and frozen sample into a mortar. Grind the tissue until the sample is a fine powder. Carefully add more liquid N_2 if needed to keep frozen.
3. Weight 500 mg of tissue into microfuge tube and add 900 µl of extraction buffer 1 (EB1) (for fruit tissue the ratio of powered tissue/EB is 1:1.8, w/v. This ratio may need to be increased to 1:3 for leaf tissue).
4. Keep on ice while preparing all samples and vortex for 30 sec.
5. Centrifuge 15 min at 4 °C and 13,000 x *g*. Remove 500 µl supernatant (clarified extract) to a fresh microfuge tube and add 850 µl of extraction buffer 2 (EB2), to give a final concentration of 35% PEG (mix well).
6. Incubate on ice for 10 min and centrifuge for 20 min at 13,000 x *g*. The supernatant is discarded.
7. Re-suspend the pellet in 100 µl of buffer 3 (B3) and keep on ice. Measure immediately PEPCK activity or the aliquots of clarified extracts can be snap frozen in liquid N_2 and stored at -80 °C for future use.

B. PEPCK activity

The activity of PEPCK is measured in carboxylation direction by following oxidation of NADH at 340 nm at 25 °C. Briefly, a continuous assay is used in which OAA produced by PEPCK is immediately reduced to malate, and this is achieved by the inclusion of malate dehydrogenase. The oxidation of NADH by malate dehydrogenase is measured at 340 nm using a spectrophotometer.

1. Accurately pipette 2-10 µl of clarified extract into a microplate well.
2. Use repeat pipetor to add 150 µl PEPCK assay mix 1 to each well and immediately place in microplate spectrophotometer. Continuously monitor NADH oxidation to NAD^+ as a decline in absorbance at 340 nm (A_{340}), taking readings every 5-10 sec for up to 5 min (the samples need to shake while reading).

3. Correct for background NADH oxidation by omitting PEP from the reaction mixture. Ensure that the decline in A_{340} (amount of NADH being oxidized; ε340 = 6,220/M/cm) is proportional to assay time and concentration of enzyme assayed. Dilution of clarified extract in extraction buffer may be necessary for samples containing abundant PEPCK activity.
   Note: One international unit (U) of enzyme activity is defined as the amount of enzyme resulting in the production of 1 µmol of product per min at 25 °C. PEPCK activity in (U/ml clarified extract) = (ΔA$_{340}$*/min x clarified extract dilution factor)/6.22. Thus, if 3.0 µl of clarified extract mixed with 150 µl of PEPCK reaction mixture yields a ΔA*$_{340}$*/min of 0.2 at 340 nm, then the PEPCK activity = (0.2 x 50)/6.22 U/ml = 1.6 U/ml.*

C. Extraction for measuring NADP-ME activity
This protocol applies to extraction of NADP-MEs from plant tissues.
1. Collect the sample and freeze immediately in liquid N_2. Store at -80 °C until use.
2. Add small amount of liquid N_2 and frozen sample into a mortar. Grind the tissue until the sample is a fine powder. Carefully add more liquid N_2 if needed to keep frozen.
3. Weight 100 mg of tissue into microfuge tube and add 200 µl of extraction buffer 4 (EB4) (for fruit tissue the ratio of powered tissue/EB is 1:2, w/v. This ratio may need to be increased to 1:3 for leaf tissue).
4. Keep on ice while preparing all samples and vortex for 30 sec.
5. Centrifuge 10 min at 4 °C and 13,000 x *g.* Transfer the supernatant to a fresh microfuge tube. Measure NADP-ME activity immediately or the aliquots of clarified extracts can be snap frozen in liquid N_2 and stored at -80 °C for future use.

D. NADP-ME activity
The activity of NADP-ME is based upon the reduction of $NADP^+$ at 340 nm at 25 °C. Briefly, a continuous assay is used in which malate is oxidized to pyruvate and CO_2, and $NADP^+$ is reduced to NADPH. The reduction of $NADP^+$ by NADP-ME is measured at 340 nm using a spectrophotometer.
1. Accurately pipette 5-10 µl of clarified extract into a microplate well.
2. Use repeat pipetor to add 100 µl NADP-ME assay mix 1 to each well and immediately place in microplate spectrophotometer. Continuously monitor $NADP^+$ reduction to NADPH as an increase in absorbance at 340 nm (A_{340}), taking readings every 5-10 sec for up to 5 min (the samples need to shake while reading).
3. Correct for background $NADP^+$ reduction by omitting L-malate from the reaction mixture. Ensure that the increase in A_{340} is proportional to assay time and concentration of

enzyme assayed. Dilution of clarified extract in extraction buffer may be necessary for samples containing abundant NADP-ME activity.

Note: One international unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of NADPH min^{-1} *under the specified conditions. NADP-ME activity in (U/ml clarified extract) = (*ΔA_{340}*/min x clarified extract dilution factor)/6.22. Thus, if 2.0 µl of clarified extract mixed with 100 µl of NADP-ME reaction mixture yields a* ΔA_{340}*/min of 1.5 at 340 nm, then the NADP-ME activity = (1.5 x 50)/6.22 U/ml = 12.5 U/ml.*

E. Bradford assay of protein concentration

1. For preparing the BSA standard curve:
   Make 1 ml stock solution of 10 mg BSA/200 ml phosphate buffered saline (PBS) (pH 7.4) and prepare a duplicate standard curve, using template below:

Final concentration (ng/µl)	Vol. (µl) BSA stock (0.05 mg/ml)	PBS (µl)	µg protein/well
0	0	200	0
6.25	25	175	1.25
12.5	50	150	2.5
18.7	75	125	3.75
25.0	100	100	5

2. Pipette 50 µl of aliquot supernatants (or its dilutions) or BSA standards to each well.
3. Add 130 µl Bradford stock solution (see Materials and Reagents section) previously dissolved in distillate water (dilution 1/5).
4. Shake briefly and wait 5 min. Determinate the OD at 595 nm. Use slope and blank obtained from the calibration to determinate the protein concentration in each extracts.

Recipes

1. Extraction buffer 1 (EB1, kept on ice)
   500 mM Bicine-KOH (pH 9.0)
   200 mM KCl
   3 mM EDTA
   5% (w/v) PEG-4000
   25 mM DTT (add freshly every time before using it)

0.4% BSA

2. Extraction buffer 2 (EB 2, kept on ice)
   500 mM Bicine-KOH (pH 9.0)
   3 mM EDTA
   55% (w/v) PEG-4000
   25 mM DTT (add freshly every time before using it)
3. Buffer 3 (EB3, keep on ice)
   10 mM Bicine-KOH (pH 9.0)
   25 mM DTT (add freshly every time before using it)
   Note: EB1, EB2, and EB3 without DTT can be kept at 4 ºC for few weeks.
4. PEPCK assay mix 1
   100 mM Hepes-KOH (pH 6.8)
   100 mM KCl
   0.14 mM NADH
   25 mM DTT (add freshly every time before using it)
   6 mM $MnCl_2$
   6 mM PEP
   1 mM ADP
   90 mM $KHCO_3$
   6 U/ml malate dehydrogenase
5. Extraction buffer 4 (EB4, kept on ice)
   100 mM Tris-HCl (pH 8.0)
   5 mM $MgCl_2$
   2 mM EDTA
   10% (v/v) glycerol
   10 mM 2-mercaptoethanol
6. NADP-ME assay mix 1 (kept on ice)
   50 mM Tris-HCl (pH 8.0)
   10 mM $MgCl_2$
   0.5 mM $NADP^+$
   10 mM L-malate

Acknowledgments

We acknowledge the excellent care of the plants by Helga Kulka (Max- Planck-Institut für Molekulare Planzenphysiologie) and Hanna Levanony (Weizmann Institute of Science). This protocol was adapted from Osorio *et al.* (2013).

References

1. Centeno, D. C., Osorio, S., Nunes-Nesi, A., Bertolo, A. L., Carneiro, R. T., Araujo, W. L., Steinhauser, M. C., Michalska, J., Rohrmann, J., Geigenberger, P., Oliver, S. N., Stitt, M., Carrari, F., Rose, J. K. and Fernie, A. R. (2011). Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *Plant Cell* 23(1): 162-184.
2. Osorio, S., Vallarino, J. G., Szecowka, M., Ufaz, S., Tzin, V., Angelovici, R., Galili, G. and Fernie, A. R. (2013). Alteration of the interconversion of pyruvate and malate in the plastid or cytosol of ripening tomato fruit invokes diverse consequences on sugar but similar effects on cellular organic acid, metabolism, and transitory starch accumulation. *Plant Physiol* 161(2): 628-643.