Malate Dehydrogenase Assay

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Malate Dehydrogenase Activity Assay

Materials:
LN_2
Glass Petri dish bottom
Scalpel with #11 blade
Flat ended forceps bent at 45°
Small aluminum foil pouch labeled
50 mM Bicine Buffer pH 8.0 (For MDH Extraction)
Triton X, Mannitol, PVPP, BSA, DTT, PMSF
50 mM Bicine Buffer pH 8.0 (For NADP-MDH Activity Assay)
NADPH, DTT, BSA, OAA, NADH
50 mM Bicine Buffer pH 9.0 (For MDH Activation)

Procedure:
Note: Make sure you use freeze clamp to assure "a fast kill" to prevent a change in malate dehydrogenase activity. The following procedure assumes use of the gas exchange fast kill machine, but a hand held freeze clamp can also be used. Sparge all buffers with N_2 overnight.

1. Get Petri dish and cool down and fill with LN_2 , have scalpel, forceps, and foil pouch nearby
2. Freeze clamp plates in LN_2 and quickly place in freeze kill apparatus
3. When you are ready, flip air switch to activate freeze clamp, leave clamp pressurized
4. Loosen the wing nut on the leaf cuvette and move cuvette panels out of the way, and cut away excess leaf material and saran wrap with the scalpel.
5. Cool forceps tips in LN_2, Release air pressure on freeze clamp and quickly move freeze clamp plats out of the way, use the forceps to place leaf disk into the petri dish with LN_2 Cut leaf into 2 pieces if need
6. Cool foil pouch in LN_2 and place leaf disk in pouch to store or to break up leaf material and place in microfuge tube.
7. Make extraction buffer in 15 ml tube according to the following table

Stock	Volume to add	Final Concentration
Bicine Buffer* 50 mM pH 8.0 deli case	10 ml	50 mM*
Triton X-100 shelf	50 µl	0.5 %
Mannitol 500 mM freezer	200 µl	10 mM
PVPP powder shelf	0.05 g	0.5 %
BSA powder frige	0.1 g	1 %
DTT 1M freezer	50 µl	5 mM
PMSF 100 mM freezer	100 µl	1 mM

* Bicine buffer also contains 30 mM NaCl, 5mM $MgCl_2$, and 2mM EDTA

8. When leaf material is in a microfuge tube grind frozen to power leaf matiral, place tube on ice and add 1 ml of extraction buffer, shake or votex tube to mix contents
9. Centrifuge for 5 minutes, place supernatent into new microfuge tube, store on ice

10. Make NADP-MDH Assay buffer in 15 ml tube according to the following formula
    1 ml 50 mM Bicince buffer pH 8.0 * X (number of samples)
    + 10 µl NADPH * X (100 µM)
    + 1 µl DTT * X (1 mM)
    + 0.0001 g BSA * X (10 % w/v)
11. Add 800 µl of the mix to each cuvette
12. Add 50 µl of sample to cuvette
13. Once you have a stable baseline add 5 µl of OAA to start the reaction
14. Now we need to fully activate MDH by reducing with DTT to do this add the following to a 500 µl microfuge tube
    + 25 µl 50 mM Bicine buffer pH 9.0
    + 25 µl DTT (166.7 mM)
    + 100 µl Sample
15. Allow the MDH to fully activate by incubating for 1 hour on the bench
16. Assay for MDH activity as before starting with step 11, remember you have diluted your sample by 1.5 X , so multiply activity by 1.5 to compare with the unactivated assay
17. Now we need to test for NAD dependent MDH activity to do this make NAD-MDH Assay buffer in 15 ml tube according to the following formula
    1 ml 50 mM Bicince buffer pH 8.0 * X (number of samples)
    + 10 µl NADH * X (100 µM)
18. Add 800 µl of the mix to each cuvette
19. Add 50 µl of sample to cuvette
20. Once you have a stable baseline add 5 µl of OAA to start the reaction
21. You can subtract this activity from the two NADP dependent MDH activities

Reactions:

OAA + $NADPH^{(absorbs\ at\ 340nm)}$ (*MDH*) $\rightarrow$ Malate + NADP

This assay was adapted from:

Scheibe R., and Stitt M. (1988) Comparison of NADP-malate dehydrogenase activation, Q_A reduction and O_2 evolution in spinach leaves. Plant Physiology and Biochemistry 26:473-481

Sharkey T. D., Badger M. R., von Cammerer S., and Andrews T. J. (2001) Increase heat sensitivity of photosynthesis in tobacco plants with reduced Rubisco activase. Photosynthesis Research 67:147-156.

Malate Dehydrogenase Activity Assay Reagents

50 mM Bicine Buffer pH 8.0 (For MDH Extraction)
In 250 ml dH_2O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.483 g NaCl, 30mM (fw 58.44)
+ 0.254 g $MgCl_2 \bullet 6H_2O$, 5mM (fw 203.3; Sigma M-2670)
+ 0.21 g EDTA, 2mM (fw 416.2; Sigma ED4SS)
pH to 8.0 using KOH

500 mM Mannitol
In 50 ml dH_2O
+ 4.56g Mannitol (fw 182.2)

Store in -20°C

1 M DTT
In 10 ml dH_2O
+ 1.54 g DTT (fw 154.2; Sigma D-5545)
Divide into 1.5 ml microfuge tubes and store in -80°C

100 mM PMSF
In 10 ml dH_2O
+ 0.174 g Phenylmethylsufonyl fluoride (fw 174.2; Sigma P-7626)
Divide into 1.5 ml microfuge tubes and store in -80°C

50 mM Bicine Buffer pH 8.0 (For NADP-MDH Activity Assay)
In 250 ml dH_2O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.11 g EDTA, 1mM (fw 416.2; Sigma ED4SS)
pH to 8.0 using KOH

10 mM NADPH
In 10 ml 100 mM carbonate buffer pH 10.3
+ 0.0833 g HADPH (fw 833.4; Sigma N-1630)
Divide into 1.5 ml tubes and store in -80°C

100 mM Carbonate Buffer PH 10.6
In 100 ml dH_2O
+ 0.848 g Na_2CO_3 (fw 105.99)
+ 0.168 g $NaHCO_3$ (fw 84.01)
pH to 10.6 using NaOH or HCl

400 mM OAA
In 10 ml dH_2O
+ 0.528 g Oxalacetic Acid (fw 132.1; Sigma O-4126)
Divide into 1.5 ml tubes and store in -80°C

50 mM Bicine Buffer pH 9.0 (For MDH Activation)
In 250 ml dH_2O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
pH to 9.0 using KOH

50 mM Bicine Buffer pH 8.0 (For NAD-MDH Activity Assay)
In 250 ml dH_2O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.11 g EDTA, 1mM (fw 416.2; Sigma ED4SS)
+ 0.254 g $MgCl_2 \bullet 6H_2O$, 5mM (fw 203.3; Sigma M-2670)
pH to 8.0 using KOH

10 mM NADH
In 10 ml 100 mM carbonate buffer pH 10.3
+ 0.0709 g HADH (fw 833.4; Sigma N-8129)
Divide into 1.5 ml tubes and store in -80°C 10 mM NADH