Phosphoenolpyruvate Carboxylase
April 7, 2005

Summary

Phosphoenolpyruvate carboxylase catalyzes the carboxylation of phosphoenolpyruvate to oxaloacetate using carbonate as a co-substrate. This protocol describes a coupled enzyme assay for determining phosphoenolpyruvate carboxylase activity.

Solutions Required

1. 50 mM Tris·HCl/$MgCl_2$ buffer (pH = 8.0):
   adjust to a pH of 8.0 with 20% NaOH.
   add enough $MgCl_2$ for a final concentration of 1 mM.

2. 0.15 M Tris·HCl buffer (pH = 8.5):
   adjust to a pH of 8.5 with 20% NaOH.

3. 0.3 M $MgCl_2$
   can be prepared in stock solution and stored at room temperature.

4. 0.6 M $NaHCO_3$
   must be prepared fresh

5. 3.0 mM NADH
   must be prepared fresh

6. 0.1 M Phosphoenolpyruvate
   must be prepared fresh

7. 15 mM acetyl CoA
   must be prepared fresh

8. Malate Dehydrogenase
   use porcine heart enzyme as purchased from Sigma, approximately 10000 U/mL

Preparation of Cell Extract

Follow general protocol **Preparation of Cell Extract**.

1. Centrifuge sufficient cells so that the volume diluted down to 5 mL would give an optical density of 20-30. For example, for a broth of OD=1, use 100 mL. For a broth of OD=10, use 10 mL.
2. After first pelletization of cells, resuspend in 5-15 mL of 4°C Tris/Mg (pH 8.0) buffer.
3. After second pelletization of cells, resuspend in 5 mL of 4°C Tris/Mg (pH 8.0) buffer, and break with French Press.

Spectrophotometer

Turn on the ultraviolet bulb on the spectrophotometer (Beckman DU50) and wait 30 minutes for warm-up. Select the kinetics-time window on the instrument. Load the method "A:/nadh". This method has a run-time of 60 s, a temperature of 37°C (or another appropriate fermentation temperature), a wavelength of 340 nm and uses 2 autosamplers.

Procedure

1. For each assay, prepare the two cocktails shown in the following table into two separate UV-translucent cuvettes, and keep them on ice.

	Volume (µL) added to:	
Solution	Control	Experimental
DI H_2O	190	140
Tris (pH = 8.5)	1000	1000
$NaHCO_3$	25	25
$MgCl_2$	25	25
NADH	50	50
acetyl CoA	50	50
Malate Dehydrogenase	10	10
PEP	0	50

2. Directly from the ice when ready to commence the assay, place the two cuvettes (each containing 1350 µL†) into the spectrophotometer holder (position #1 for control, position #2 for experimental).
3. Wait 10 minutes to allow the temperature of the solutions in the cuvettes to equilibrate.
4. "Blank" and then depress "Read Samples" on the monitor.
5. Simultaneously add 150 µL† of the cell extract to the cuvettes.
6. To mix solutions, immediately and simultaneously aspirate and dispense the contents of the cuvettes with a pipettor. Mix the solutions in this way ten times. (Count!)
7. Promptly depress “start” on the monitor.
8. Record the rates for the two (control and experimental) cuvettes.

† Dilution of the cell extract may be adjusted so that change in absorbance is between about 0.05 and 0.7 AU in one minute. This dilution should be accomplished externally in a microcentrifuge tube (for example, by adding 50 µL of cell extract to 950 µL DI water to achieve a dilution of 20). The volume of 150 µL should always be used in the enzyme assay mixture.

Calculation of Activity

One unit (U) of phosphoenolpyruvate carboxylase activity is defined as the amount of enzyme required to produce 1.0 μmole of oxaloacetate in one minute.

1. $dA/dt\ (min^{-1}) = [Rate]_{experimental} - [Rate]_{control} = dA/dt$

2. $$\text{Activity} = \frac{1000 \times TV \times D \times dA/dt}{\varepsilon \times V \times CF}$$

Activity: Volumetric Activity (U/L)
TV: Total volume in cuvette (1000 μL)
D: Dilution of the cell extract. (For example, if 50 μL of cell extract were add to 950 μL DI water prior to using a volume of cell extract in the assay, then D=20)
V: Volume of cell extract used (150 μL)
ε: Molar extinction coefficient for NADH (6.22 L/mmol for a path length of 1.0 cm)
CF: Concentration Factor of cell extract (For example, if a 100 mL sample is concentrated to a 2 mL volume for the French Press, then CF=50)

3. $$\text{Specific Activity} = \frac{Activity}{Protein\ Concentration} 1$$

Activity: Volumetric Activity, as calculated in #2 above (U/L)
Protein Concentration: Protein concentration, as calculated in protocol **Total Protein Concentration** (mg/L)
Specific Activity: (U/mg protein)

Reference

P. Maeba, B. D. Sanwal (1969) Phosphoenolpyruvate carboxylase frm *Salmonella typhimurium* strain LT2, Methods in Enzymology 13, 283-288.

J. L. Cánovas, H. L. Kornberg (1969) Phosphoenolpyruvate carboxylase from *Escherichia coli*, Methods in Enzymology 13, 288-292.