PEP Carboxykinase
April 5, 2005

Summary

PEP carboxykinase catalyzes the carboxylation of PEP to oxaloacetate with the generation of ATP. In this coupled assay, the ATP generated by this reaction acts as a substrate in the conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate via the enzyme 3-phosphoglycerate phosphokinase. The 1,3-diphosphoglycerate so generated is then converted to glyceraldehyde 3-phosphate via the enzyme glyceraldehyde 3-phosphate dehydrogenase and NADH, which is supplied in the reaction mixture. The disappearance of NADH is what is actually measured.

Solutions Required

1. 500 mM tris-HCl pH = 6.6

2. 350 mM $NaHCO_3$
   can be prepared in stock solution and stored at room temperature.

3. 160 mM $MgCl_2$
   can be prepared in stock solution and stored at room temperature.

4. 6 mM NADH
   can be prepared in stock solution and stored at -20°C

5. 20 mM dithiothreitol (DTT)
   can be prepared in stock solution and stored at -20°C

6. 0.2 M ADP
   can be prepared in stock solution and stored at -20°C

7. 36 mM 3-phosphoglycerate
   must be prepared fresh

8. 50 mM PEP
   must be prepared fresh

9. 3-Phosphoglycerate phosphokinase (PGPK)
   suspension as purchased from Sigma (P7634)
   prepare just prior to use a solution which contains 4 U/100µL. A 100 µL will be needed for each assay (50 µL for experimental and 50 µL for control).

10. Glyceraldehyde 3-phosphate dehydrogenase (GPDH)
    suspension as purchased from Sigma (G9263, 92 U/mg)
    prepare just prior to use a solution which contains 4 U/100µL. A 100 µL will be needed for each assay (50 µL for experimental and 50 µL for control).

Preparation of Cell Extract

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

1. Centrifuge sufficient cells so that the volume diluted down to 2 mL would give an optical density of 40-50. 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 100 mM tris-HCl buffer.
3. After second pelletization of cells, resuspend in 2 mL of 4°C 100 mM tris-HCl buffer, and break with French Press.

Spectrophotometer

Turn on the ultraviolet bulb on the spectrophotometer (Beckman DU50) and wait 10 minutes for warm-up. Select the kinetics-time window on the instrument. Load the method "A:/ppc". This method has a run-time of 60 s, a temperature of 37°C, 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	200	100
tris	200	200
$NaHCO_3$	100	100
$MgCl_2$	100	100
DTT	50	50
ADP	50	50
3-phosphoglycerate	50	50
PEP	0	100
NADH	50	50
PGPK	50	50
GPDH	50	50

2. Directly from the ice when ready to commence the assay, place the two cuvettes (each containing 900 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 the spectrophotometer using water (“Read samples”).
5. Simultaneously add 100 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.

† Our experience is that the cell extract has to be diluted by a factor of about 20 when using overexpressed PCK. This dilution should be accomplished in a microcentrifuge tube (for example, by adding 50 µL of cell extract to 950 µL DI water). The volume of 100 µL should always be used in the enzyme assay mixture.

Calculation of Activity

One unit (U) of PEP carboxykinase activity is defined as the amount of enzyme required to produce 1.0 µmole of oxaloacetate in one minute. This quantity is proportional to the amount required to generate 1.0 µmole ATP, and to the amount required to generate 1.0 µmole NADH via this coupled enzyme assay.

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 (100 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. $$\text{Specific Activity} = \frac{Activity}{Protein\ Concentration}$$

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. Kin, M. Laivenieks, C. Vieille, J. G. Zeikus (2004) Effect of overexpression of *Actinobacillus succinogenes* phosphopyruvate carboxykinase on succinate production in *Escherichia coli*, Appl.

Environ. Microbiol. 70(2):1238-1241.