Pyruvate Carboxylase

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Summary

Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate using ATP and carbonate as co-substrates. This protocol describes a coupled enzyme assay for determining pyruvate carboxylase activity. The oxaloacetate generated by the action of pyruvate carboxylase reacts with acetyl CoA via citrate synthase (both in excess). The free CoA generated by this second reaction is the species that actually causes the signal by its reaction with DTNB.

Solutions Required

1. 1.0 M Tris·HCl pH = 8.0

2. 0.5 M $NaHCO_3$
   can be prepared in stock solution and stored at room temperature.

3. 0.1 M $MgCl_2$
   can be prepared in stock solution and stored at room temperature.

4. 1.0 mM Acetyl CoA
   can be prepared in stock solution and stored at -20°C

5. 0.1 M Pyruvate
   can be prepared in stock solution and stored at -20°C

6. 0.1 M ATP
   can be prepared in stock solution and stored at -20°C

7. 0.0039 g DTNB in 1.0 mL 100% ethanol
   must be prepared fresh

8. Citrate Synthase
   solution as purchased from Sigma (C3260), approximately 1000 U/mL

Preparation of Cell Extract

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

1. Centrifuge sufficient cells so that the volume concentrated to 2 mL would give an optical density of about 25. For example, for a broth of OD=0.5, use 100 mL for the enzyme assay. For a broth of OD=10, use 5-10 mL.
2. After first pelletization of cells, resuspend in 5-15 mL of 4°C 100 mM tris·HCl pH 8.0.
3. After second pelletization of cells, resuspend in 2 mL of 4°C 100 mM tris·HCl pH 8.0, and break with French Press. Centrifuge again to remove the cell debris.

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:/pyc". This method has a run-time of 60 s, a temperature of 30°C, a wavelength of 412 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	530	480
tris·HCl	90	90
$NaHCO_3$	100	100
$MgCl_2$	50	50
Acetyl CoA	100	100
DTNB	25	25
Citrate Synthase	5	5
ATP	50	50
Pyruvate	0	50

2. Directly from the ice when ready to commence the assay, place the two cuvettes (each containing 950 µL†) into the spectrophotometer holder (position #1 for control, position #2 for experimental). Use cuvette lid caps to mix 3 or 4 times then insert in instrument.
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 50 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 50 µL should always be used in the enzyme assay mixture.

Alternatively, the 950 µL reaction mixture can be *added to* the 50 µL of the cell extract in the cuvette. This approach is a better way to mix the cell extract with the reactants, but additional steps must be taken to equilibrate the temperature of the reactants to 30°C before mixing.

Calculation of Activity

One unit (U) of pyruvate 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 (50 µL)
ε:	Molar extinction coefficient for reduced DTNB (13.6 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

Payne, J., Morris, J.G., Pyruvate carboxylase in *Rhodopseudomonas spheroides*, J. Gen. Microbiol., 1969, 59, 97-101.

Note: The original assay calls for PBS buffer. We have found that tris·HCl buffer works well, is more readily available in our lab, and causes less background activity.