Citrate-Synthase Assay Procedure

Introduction

Citrate synthase is a pace-maker enzyme in the Krebs cycle (citric acid cycle or tricarboxylic acid cycle, TCA). Citrate synthase, CS, has a molecular weight of 51,709 Da, with gene map locus 12q13.2- q13.3. CS is localized in the mitochondrial matrix, but is nuclear encoded, synthesized on cytoplasmic ribosomes and transported into the mitochondrial matrix. CS, therefore, is commonly used as a quantitative marker enzyme for the content of intact mitochondria (Holloszy et al 1970; Willimas et al 1986; Hood et al 1989). Proliferation of mitochondria in pathological states is sometimes associated with an increase in CS activity per cell, but CS activity in a specific tissue is frequently constant when expressed per mitochondrial protein or per mt-respiratory capacity (Renner et al 2003). Mitochondrial, cellular or tissue respiration, therefore, may be expressed per CS activity for specific applications (Kuznetsov et al 2002; Renner et al 2003; Hütter el al 2004).

Enzymatic reaction catalyzed by citrate synthase:

Acetyl-CoA + oxalacetate + H2O -> citrate + CoA-SH

Citrate synthase assay coupled to enzyme reaction (above):

CoA-SH + DTNB -> TNB + CoA-S-S-TNB

Absorbance and enzyme activity: The optical density, **OD**, of a liquid sample is related to the absorbance, **A**, by the optical path length, [cm]-1. **A** is a dimensionless number. The path length is fixed by the dimension of the spectrophotometric cuvette. The molar extinction coefficient of the absorbing substance **B, eB** [mM-1 ×cm-1], is specific for the compound studied at a particular wavelength. Absorbance increases with molar concentration, **cB** [mM], in the solution contained in the cuvette. The rate of increase of the absorbance is the slope, **rA = dA/dt**, which is proportional to enzyme activity.

$$OD = \frac{A}{I} = \varepsilon_B * c_B$$

$$rA = \frac{dA}{dt} \sim Enzyme\ Activity$$

The reaction product **TNB** (thionitrobenzoic acid) is the absorbing substance **B** with intense absorption at 412 nm. Therefore, the working wavelength is **412 nm**. The absorbance increases linearly with time, up to 0.6-0.8 units of absorbance (over 200 s of measurement). The enzyme activity is not affected by up to 1% Triton X-100. When CS activity is used as a marker, it is not critical to choose a physiological temperature. A constant reference temperature has to be applied for comparability of measurements. Measurements are frequently performed at room

temperature, but more commonly at 30 °C (Hütter et al 2004; Kuznetsov et al 2002; Renner et al 2003; Trounce et al 1989).

Materials

- ☐ UV/visible spectrophotometer with temperature controlled cuvette holder
- ☐ 1mL cuvettes (x2)
- ☐ 1.5mL Eppendorf tubes (x1000)
- ☐ Microbalance
- ☐ parafilm
- ☐ 1L glass beakers (x2)
- ☐ pH meter
- ☐ thermometer
- ☐ distilled water
- ☐ stir rod
- ☐ 10 µl Hamilton syringe
- ☐ Medium (MiR06/MiR07)
- ☐ 37% HCl
- ☐ Tris (hydroxymethyl) aminomethane C4H11NO3
- ☐ Triethanolamine (8g)
- ☐ EDTA (Ethylenediaminetetra acetic acid disodium salt dehydrate)
- ☐ Triton X-100 (10mL)
- ☐ acetyl CoA (25 mg)
- ☐ Oxalacetic acid C4H4O5 (>6.6 mg)
- ☐ DTNB (5,5′-Dithiobis(2- nitrobenzoic acid), Ellman's reagent (2mg)
- ☐ Frozen tissue samples (freeze dried or -80°C)
- ☐ Citrate synthase, CS (8.6 mg prot./ml)
- ☐ Ika T digital ULTRA-TURRAX (or similar)
- ☐ 15mL falcon tubes
- ☐ Liquid Nitrogen
- ☐ Ice + Bucket

Procedure

Reagents to prepare every month new and store at 4 °C (fridge temp)

Tris-HCl buffer (1.0 M, pH 8.1): 2.4228 g Tris/20 ml a.d., adjust to pH 8.1 with 37% HCl (ca. 100 µl/20 ml).

Tris–HCl buffer (0.1 M, pH 7.0): 2 ml 1M Tris-HCl buffer, pH 8.1+ 15 ml a.d. Adjust pH to 7 with concentrated HCl and fill up to 20 ml with a.d.

Triethanolamine-HCl buffer (0.5 M, pH 8.0) + EDTA (5 mM): 8.06 g triethanolamine/100 ml a.d., adjust pH with 37% HCl, add 186.1 mg EDTA. pH does not change after addition of EDTA. Triethanolamine is viscous. Weigh in a beaker on the balance.

Triton X-100 (10% solution): Reagent solution is 100%, add 90 ml a.d. to 10 g (ca. 10 ml) Triton X-100. Triton X-100 is viscous and sticky. Weigh on balance in a beaker and dissolve by stirring.

Reagents to prepare and store at -20 °C

12.2 mM acetyl-CoA, 25 mg acetyl CoA + 2.5 ml a.d., make aliquots of 250 µl and store at -20 °C. Store on ice during measurement, freeze it again after the experiment.

Reagents to prepare everyday (cannot store)

Triethanolamine-HCl-buffer (0.1 M, pH 8.0): 1 ml of 0.5 M triethanolamine-HCl-buffer of pH 8.0 + 4 ml a.d.

Oxalacetate (10 mM, pH 8.0): 6.6 mg oxalacetate + 5 ml of 0.1 M triethanolamine-HCl-buffer of pH 8.0.

DTNB (1.01 mM, pH 8.1): 2 mg DTNB + 5 ml of 1 M Tris-HCl-buffer of pH 8.1.

Sample Preparation:

1. During measurements, store the sample on ice (4°C). CS activity of cells is stable during storage (a few hours) on ice.
2. Whole tissue slices have to be homogenated with an Ultra Turrax (T10 basic; IKA) for 20 to 30 s at level 4 before CS measurement.
3. Due to very high CS activity of isolated mitochondria, the suspension for measurement can be prepared by dilution (1:3 to 1:10) of a frozen stock mitochondrial suspension (-80 °C; usually ca. 50 mg of mitochondrial protein per ml).
4. Immediately after thawing, add 20 µl of mitchondrial suspension to 180 µl of 0.1 M Tris-HCl buffer, pH 7.0 (RT). During measurement, store on ice. Freeze stock suspension again. 10-25 µl mitochondrial suspension (5 mg/ml) is used for each spectrophotometric measurement.

5. For typical cells at 1-2.106 cells/ml, take replicates of 110 µl samples into Eppendorf tubes, freeze in liquid nitrogen, and store until measurement.

CS Standard prep

6. As a standard, commercial citrate synthase is diluted 1:500 in 0.1 M Tris-HCl buffer, pH 7.0 (RT). Accurate dilution is critical and is achieved by adding 2 µl of CS standard (using a 10 µl Hamilton syringe) to 998 µl of buffer.
7. Starting with a protein concentration of 8.6 mg×cm-3 in the undiluted CS standard, this yields a final protein concentration of 0.0172 mg×cm-3 in the sample, of which 5 µl are added to a volume of 995 µl of reaction mix (1:200). At the end, the dilution is 1:100.000. Always use freshly diluted enzyme.

Spectrophotometry

8. The spectrophotometer has to be switched on about 10 min before measurement. First, before turning on the photometer, remove all cuvettes from the cuvette holder, then switch on the power supply of the spectrophotometer, the computer, and the monitor.
9. Set up a kinetic program at 412 nm, this will vary depending on the brand of spectrophotometer
10. Add 1 ml a.d. into a glass cuvette and insert the cuvette into the spectrophotometer at position 1, run blank
11. Add all reaction components (except for the 10 mM oxalacetate solution) in the given sequence (like this the components are gently mixed and the reaction mixture turns yellow)

Component	Volume added (uL)	Final Concentration
10% Triton X-100	25 uL	~0.25%
Acetyl CoA	25 uL	~0.31 mM
1.01 mM DTNB	100 uL	~0.1 mM
Volume CS standard	5 uL	
Volume Medium	10 uL	
Volume Cell suspension	100 uL	
Distilled H2O	800 uL-Volume X	

Oxalacetate	50 uL	~0.5 M

12. Prepare all components (triton X-100, Acetyl CoA, DTNB, V, H2O) in a glass cuvette except oxaloacetate.
13. Add oxaloacetate into the cuvette, seal with parafilm and your thumb. Swivel gently 3 times. Remove parafilm and place the cuvette at position 1 (front).
14. The first 3 samples measured should be: MiR06 (10 µl) , CS standard (5 µl), CS standard replicate (5 µl)
15. Click Start and record absorbances and absorbance rates (slope) in spreadsheet

Data analysis: Calculation of specific CS activity

16. The rate of concentration change of the absorbing compound B in the cuvette, dcB/dt, is calculated from the rate of the absorption change:

$$\frac{dc_b}{dt} = \frac{r_A}{l * \varepsilon_B} = \frac{\frac{dA}{dt}}{l * \varepsilon_B}$$

17. The reaction flux per unit volume, JV, in the cuvette is:

$$J_v = \frac{dc_B}{dt} * v_b^{-1}$$

18. The specific enzyme activity is proportional to the experimental reaction flux and inversely proportional to the dilution factor, Vsample/Vcuvette and to the mass concentration, r [mg×cm-3] or cell density [106 ×cm-3] in the sample, Vsample. The specific enzyme activity, n, is the velocity of the enzyme-catalyzed step per unit sample, measured under experimental incubation conditions with a saturating substrate.

$$v\ (per\ unit\ sample) = \frac{r_A}{l * \varepsilon_B * v_B} * \frac{V_{cuvette}}{V_{sample} * \rho}$$

v, is the specific activity of the enzyme expressed per mg protein or per million cells [IU/mg protein or IU/106 cells], depending on r. Enzyme activity is frequently expressed in international units, IU [µmol/min]. 1 IU of CS forms 1 µmol of citrate per min.
rA = dA/dt, is the rate of absorbance change [min-1].
Optical path length (= 1 cm).
eB: Extinction coefficient of B (TNB) at **412 nm** and pH 8.1 = 13.6 mM-1 ×cm-1 = (13.6 mmol·dm-3) -1 ·cm-1 .

vB, Stoichiometric number of B (TNB) in the reaction (= 1).
Vcuvette: Volume of solution in the cuvette (= 1000 µl).
Vsample: Volume of sample added to cuvette (5 - 100 µl).
p: Mass concentration or density of biological material in the sample, Vsample: (protein concentration: mg×cm-3 ; cell density: 106 ×cm-3).

References:

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