Protoporphyrinogen Oxidase Activity Assay

PROTOPORPHYRINOGEN OXIDASE

Cultured cell homogenate

1. Resuspend the pellet of PBS-washed cells in about three volumes of 50mM potassium phosphate pH 7.4 (KPi).
2. Sonicate (homogenize) while in an ice bath at the lowest practicable power setting for 3 cycles x 5 sec at 50% duty (2.5 sec on, 2.5 sec off).
3. Determine the protein concentration and adjust to 4mg/mL with more KPi.

Preparation of protoporphyrinogen

1. Prepare about 1.5mL of 1mM PPIX in an aqueous solution also containing 10mM KOH and 20% v/v ethanol in a glass test tube with a magnetic flea.
2. Put in approximately 150mg of 20% sodium in mercury amalgam (Acros Organics, Bridgewater, NJ). Cover the tube but with allowance for needle introduction and exhaust of gases.
3. Minimize exposure to light for the rest of this procedure.
4. Stir the mixture vigorously, with occasional strong shaking, for about half an hour under blowing argon gas or until all the fluorescence is gone, as monitored with minimal time exposure to a UV lamp.
5. While in an argon-filled AtmosBag (Sigma-Aldrich, St. Loius, MO), remove the solids with a syringe filter. Measure the volume of the resulting protoporphyrinogen solution.
6. Add 9 volumes of raw incubation buffer containing 120 mM Tris base, 2.5 mM EDTA free acid, 100 mM ascorbic acid and 0.12% Triton X-100.
7. Aliquot into 1.7-mL microfuge tubes and put in argon-inflated ziplock bags. Take out of the AtmosBag and store frozen.
8. Strictly follow published methods to store and then dispose of mercury.

Protoporphyrinogen oxidase reaction

1. Adjust the pH of the substrate protoporphyrinogen solution to around 8.5 with 3M HCl before use. Use pH paper indicators.
2. Add 90 µL of the pH adjusted substrate solution 10µL of sample containing 4 mg/mL protein/mL, in a 1.7mL microfuge tube. (There is no need to vortex the tubes, as this will enhance autooxidation. There is also more than enough oxygen substrate above the liquid in the tubes for the duration or the reaction.)
3. Pair each live sample with a blank, which is sample that has been heated in a boiling water bath for 10 min.
4. Incubate at 37˚C for 10 min.
5. Stop the reaction with 400µL of 30 % v/v DMSO in methanol.
6. Centrifuge at 16000xg for 10 min.
7. Immediately inject the supernatant into the UPLC, the blank and the live samples one after the other.

Quantification of product protoporphyrin IX.

1. Inject 10µL of supernatant solution of porphyrins into a Waters Acquity UPLC (ultra performance liquid chromatography) system, which includes a binary solvent manager, sample manager, photodiode array detector (PDA), fluorescence detector (FLR), column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 100 mm column.
2. Set the FLR at 404 nm excitation and 630 nm emission to measure fluorescence of protoporphyrin IX (PPIX). Use the PDA to verify the absorption spectrum of PPIX peak.
3. Keep the sample chamber dark and at ambient temperature.
4. Solvent A is 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol. Keep the flow rate constant at 0.40 mL per minute and maintain the column at 60˚C for the total run time of 6 min. Use the following successive linear gradient settings for run time in minutes versus Solvent A: 0.0, 80%; 1.5, 1%; 3.5, 1%; 4.0, 80%.
5. For standards, use solutions of known concentrations of PPIX also dissolved in 30%DMSO in ethanol. Subtract the blank PPIX readings from every corresponding live sample.

Major reference:

Li, F., CK Lim and TJ Peters. An h.p.l.c. assay for protoporphyrinogen oxidases activity in rat liver. Biochem. J. (1987) 243, 863-866.

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