Method Sheet 39

Determination of enzyme inhibitor class by Lineweaver-Burk plot

Overview

This method sheet explains how to test what type of inhibitor(s) your hit natural extracts may contain with respect to the enzyme β-galactosidase. Establishing this is a key step in the drug discovery process, as most drugs can be classified as competitive, non-competitive or uncompetitive inhibitors of their targets. This is achieved by measuring the initial reaction rate of an enzyme incubated with a series of different substrate concentrations, in the presence or absence of the inhibitor (in this case, the hit extract). Plotting the results from this type of experiment on a Lineweaver Burk plot allows us to tell what type of inhibitor is present in the extract by examining the ways the lines intersect on the chart.

Reagents

- Recombinant beta-galactosidase enzyme (1 mg/ml, frozen aliquot)
- Ortho-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg, dry powder)
- One or more hit natural extracts at 10 mg/ml in DMSO
- Phosphate buffer (50 mM, pH 6.8, see notes section below for how to prepare)

Equipment

- Clear plastic 96-well microplate(s) with lids (do not need to be sterile)
- Pipette tips (compatible with each pipette)
- Plastic reservoir(s) to dispense ONPG substrate
- Multichannel pipette (8- or 12-channel) capable of dispensing 1 - 10 µl
- Multichannel pipette (8- or 12-channel) capable of dispensing 100 - 200 µl
- Suitable waste stream for disposal of spent plates and reactions
- Microplate reader capable of measuring absorbance of 96-well plates at 420 nm (450 nm is also suitable)

Method: Part 1 - Preparing the substrate dilutions

1) Defrost the hit *Phytotitre* extract vial(s) and a control vial of sterile DMSO alone.
2) Add 60 µl of DMSO to 1,440 µl of phosphate buffer in a microtube and mix well.
3) Add 60 µl of hit extract to 1,440 µl of phosphate buffer in a second microtube and mix well.
4) Aliquot 50 µl of the DMSO solution to every well of the plate from well B2 to D9, as shown in the plate map below.
5) Aliquot 50 µl of the Extract solution to every well of the plate from well E2 to G9, as shown in the plate map below.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml			
C		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	+1% DMSO		
D		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml			
E		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml			
F		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	+100 µg/ml Extract		
G		0 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml			
H												

6) Add 4 mg ONPG compound (the substrate) to 1 ml of phosphate buffer (see notes section below for how to prepare) to prepare a stock of substrate at 4 mg/ml.
7) Label 8 1.5 ml microtubes with the following ONPG concentrations: 0, 2, 4, 8, 16, 32, 64 and 128.
8) Add 400 µl of phosphate buffer into tubes 0 through to 64.
9) Add 697.6 µl of phosphate buffer into the 128 tube.
10) Add 102.4 µl of stock ONPG (the solution at 4 mg/ml) to the phosphate buffer in the 128 tube and mix well by gently pipetting up and down.
11) This gives a concentration of 512 µg/ml extract in the tube labelled 128, but that concentration will halve to 256 µg/ml when we add 50 µl of this suspension to the 50 µl of extract already in the well, and then in the next step it will be halved again to 128 µg/ml (the final concentration) when we add 100 µl of enzyme.
12) Now perform serial doubling dilutions of the ONPG substrate by transferring 400 µl of the suspension from the 128 tube into the 64 tube, mixing gently by pipetting several times, then doing the same to the next tube until reaching the 2 tube, but do not put any substrate in the tube labelled 0, this must contain phosphate buffer only.
13) Add 50 µl of each substrate dilution to the wells shown on the plate map above (i.e. each substrate concentration will go into one column of 6 wells).
14) The plate now contains both the substrate and the extract (inhibitor), but the reaction will not start until we add the enzyme.

Method: Part 2 - Adding enzyme to start the assay

1) Aliquot 15 ml of phosphate buffer into a clean tube, and add 3 µl of 1 mg/ml recombinant β-galactosidase enzyme (final concentration 200 ng/ml, ~0.1 U/ml).
2) Mix well, then pour the enzyme solution into a plastic reservoir.
3) Use a multi-channel pipette to aliquot 100 µl of the enzyme solution into every well of the plate that contains substrate according to the plate map shown above.
4) As soon as pipetting of enzyme into the plate is complete, use a microplate reader to measure the absorbance of the plate at 420 nm (or 450 nm if that wavelength is not available) to obtain the baseline (time = 0 h) measurements.

5) Take a note of the time, then place the assay plate in a static 37°C incubator to allow the reaction to progress.
6) Incubate the plate at 37°C for 30 minutes (or shorter or longer if necessary depending on how quickly the reaction progresses, check the plate occasionally and use the timepoint at which a clear yellow colour becomes visible at the highest concentration of substrate).
7) **Crucial**: Because we are using a single measurement as a proxy for the initial reaction rate, we cannot allow the reaction to progress for too long before reading the plate, if the assays begin to plateau, the assay will be unreliable, so make sure to read the plate before the yellow colour becomes very dark at the highest substrate concentrations.
8) Use a microplate reader to measure the absorbance of the plate at 420 nm (or 450 nm if not available) a second time.
9) Proceed to analysis of the data by plotting a Lineweaver-Burk plot according to the advice given in Method sheet 40.

Notes

- Prepare phosphate buffer as follows: place ~160 ml distilled water (dH_2O) in a beaker, add 1.70 grammes anhydrous Na_2HPO_4, 0.96 grammes anhydrous NaH_2PO_4, stir until fully dissolved, pH to 6.8 using HCl or NaOH, make up to 200 ml by adding dH_2O.
- This method shows how to test the relative inhibitory activity of one hit extract, if you are examining more than one hit extract, you will have to prepare and challenge more than one 96-well plate (one plate per extract).
- The final concentration of extract tested in this experiment is 100 µg/ml, and the final concentration of DMSO (the negative control) is 1%.

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