MBTH Assay

Introduction

The 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay quantifies the amount of reducing ends in a sample. When SmChiA and PpChi perform hydrolysis, they generate a new reducing end. As a result, by taking samples of the SmChiA/PpChi reaction mixture at different time points and performing the MBTH assay, an overview of the reaction progress can be made, enabling determination of kinetic constants.

Materials

- Enzyme of Interest
  - 34mg/L SmChiA
  - 34mg/L PpChi
- Substrate
  - Chitin from shrimp shells (for SmChiA)
  - 12M HCl
  - Chitosan (for PpChi)
  - 1% v/v acetic acid
- N-Acetyl-Glucosamine
- 50mM Sodium Acetate pH 6.0
- Assay Reagents
  - 0.5M NaOH
  - 3g/L MBTH
  - 1g/L DTT
  - 0.5% $FeNH_4(SO_4)_2.12H_2O$
  - 0.5% sulfamic acid
  - 0.25M HCl

Procedure

Preparation

1. In a fume hood, create colloidal chitin substrate by dissolving chitin powder in 12M HCl.

   Weigh 2g chitin powder in a 100mL beaker
   Add 20mL 37% HCl and stir thoroughly to break up lumps
   Sit the beaker in an ice bath

Stir every 5 minutes for 30 minutes total
Pour the acid slurry gradually into 400mL of chilled distilled water in a 1L beaker with magenetic stirring
Stir for 20 minutes to produce the colloidal chitin precipitate
Centrifuge in 50mL Falcons at 4000g 15 min and decant.
Resuspend with 45mL MilliQ water. Repeat centrifuge and decant until the supernatant reaches pH 7.
Centrifuge and resuspend in 70mL 50mM sodium acetate pH 6
Makes 70mL ~20g/L (2% w/v) colloidal chitin (assuming no yield losses)

2. Create chitosan substrate by dissolving chitosan powder in 1% v/v acetic acid.

   Begin stirring 20mL of 1% acetic acid using a magnetic stirrer at 40 degrees Celsius
   Sprinkle in 200mg chitosan slowly
   Wait until fully dissolved to have created 20mL 10gL chitosan

3. Create a sample preparation mixture by mixing equal amounts 3g/L MBTH and 1g/L DTT.

   Use immediately - degradation of DTT occurs immediately.

4. Create a colour development solution with the following components; 0.5% $FeNH4(SO4)2.12H2O$, 0.5% sulfamic acid, and 0.25M HCl.

Reaction

5. Incubate the enzyme (34mg/L in 50mM sodium acetate pH 6.0) with its substrate (1g/L) at 40 degrees Celsius (based off of kinetic model).

   Note that it is incredibly hard to have a homogenous chitin concentration. Regardless, the following volumes were used:
   - 1826uL sodium acetate
   - 100uL chitin/chitosan solution
   - 74uL of enzyme solution

6. Take 200uL samples at relevant time points (from literature, it is recommended to take them at 0, 2, 5, 10, 15, 30, 60, and 80 minutes).

7. Immediately add the sample to 200uL of 0.5M NaOH to denature the enzyme (ending the reaction) and convert all reducing ends to aldehydes.

Assay

8. Mix the sample with 200uL of sample preparation mixture and seal the tube. Heat at 80 degrees Celsius for 15 minutes. This causes MBTH to form an azine with the aldehyde.

   Note that this step must produce leftover MBTH for the next step to work. As a result, the assay is only valid for concentrations of reducing ends up to 100uM.

9. Add 400uL of colour development solution and mix by inverting. The solution converts excess MBTH to a cation. This cation reacts with the azines to produce a blue colour change.

10. Once cooled to room temperature, measure the sample absorbance at 620nm OD. Use samples of 200uL.

    Fundamentally, the amount of cleavage events corresponds to the increase in reducing ends. The amount of reducing ends corresponds to the amount of aldehydes. The amount of aldehydes corresponds to the amount of azines. the amount of azines corresponds to the intensity of the blue colour.

11. Compare the sample results over time to a standard curve made using N-Acetyl-Glucosamine. This enables creation of a product over time graph.

    This should be 6 tubes - 0, 20, 40, 60, 80, 100uM. To do this:
    1. Dissolve 110mg of glucosamine in 10mL 50mM sodium acetate pH 6 to get 50mM
    2. Dilute the 50mM solution to 500uM by adding 50uL stock to 4950uL sodium acetate buffer
    3. Create the standard curve using 500uM stock as follows,
    100uM: 200uL stock, 800uL buffer
    80uM: 160uL stock, 840uL buffer
    60uM: 120uL stock + 880uL buffer
    40uM: 80uL stock + 920uL buffer
    20uM: 40uL stock + 960uL buffer
    0uM: 1000uL buffer
    4. Use these like samples used for the assay (NaOH, sample prep mixture, heat, colour development mixture)
    5. Use in a microwell plate