Investigation of Bi-Substrate Enzyme Kinetics for the Introductory Biochemistry Lab

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Abstract

Glutathione *S*-Transferase (GST) is a bi-substrate enzyme that plays an important role in drug detoxification. The enzyme's activity is measured by colorimetric methods using 1-chloro-2,4-dinitrobenzene (CDNB), which is well suited to quantitative monitoring in real time using visible absorbance spectrometry. The conditions of this assay as previously employed in the Introductory Biochemistry Lab at Sacred Heart University were poorly suited to large scale kinetic analysis. Here, we are developing a tractable and highly reproducible large scale kinetic assay that is based on the CDNB method. We are also optimizing the conditions of this assay so that it can be applied within the educational environment and we are determining the optimal conditions for kinetic analysis to investigate the kinetic mechanism of GST. As a *transferase,* GST adopts an enzymatic mechanism that is thought to require at least two substrates: glutathione, and the target compound to which it is transferred (various drugs, or other metabolites). Over the course of our project, we will seek to investigate the enzymatic mechanism by which GST transfers glutathione onto its conjugation target, which in our research is CDNB.

Introduction

Background:

- GST plays an important role in drug detoxification
- The enzyme can be readily expressed and purified in an active form from *E. coli*
- GST activity can be measured by colorimetric methods
- Glutathione transfer neutralizes electrophilic sites within many toxic compounds, rendering them more water-soluble and facilitating subsequent metabolic processing and excretion

Prior work at Sacred Heart University:

- Polyhistidine tagged GST was purified using immobilized metal affinity chromatography (IMAC) in the first-semester biochemistry lab
- The enzyme's purity and concentration was evaluating using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford assays
- Activity was demonstrated using a color assay, which provided a measure of glutathione conjugation onto CDNB
- Conditions of the CDNB assay as previously employed were poorly suited to detailed kinetic analysis

Research goals:

1. To develop a tractable, reproducible kinetic assay for GST activity
2. To optimize conditions of this assay for application within the second semester biochemistry lab
3. To apply this optimized assay to investigate the kinetic mechanism of GST

Biochemistry at Sacred Heart University

Purification of 6xHis-GST

IMAC Purification Workflow

Culture cells expressing His-tagged fusion protein
⇓
Harvest cells by centrifugation
⇓
Lyse cells by freeze fracture, chemical or mechanical disruption
⇓
Clear insoluble materials from cell lysate by centrifugation
⇓
Apply clarified cell lysate to IMAC column
⇓
Rinse nonspecific proteins from IMAC column
⇓
Elute His-tagged proteins from IMAC column using imidazole
⇓
Concentrate purified protein and remove imidazole
⇓
Store and characterize

SDS-PAGE Gel of 6xHis-GST

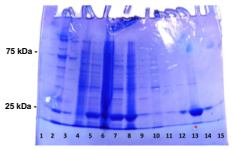


Figure 1. SDS-PAGE analysis of 6xHis-GST. Lane 1: empty, 2: protein size standard, 3: empty, 4: uninduced cell culture, 5: induced cell culture, 6: crude lysate, 7: crude lysate-supernatant, 8: crude lysate-pellet, 9: unbound IMAC flowthrough, 10-12: IMAC wash, ***13: 6xHis-GST**, 14 and 15: empty.

The bands in the gel validate the process of IMAC purification; the purified 6xHis-GST appeared at approximately 26 kDa while the literature value was 26.7 kDa (Gadagbui et al., 1999).

Determination of Enzyme Concentration

Bradford Assay

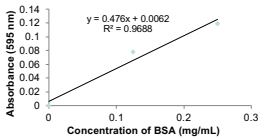
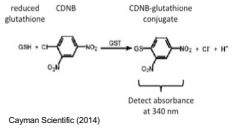


Figure 2. Determination of 6xHis-GST concentration by Bradford Assay. Serial dilutions of BSA were prepared and the absorbance of each was measured at a wavelength of 595 nm to create a calibration curve. The curve was then used to determine the concentration of enzyme.

The absorbance of the 6xHis-GST sample was 0.153, corresponding with enzyme concentration of 6.2 mg/mL in approximately 1.0 mL in storage buffer.

Analysis of GST Activity

Colorimetric assay principle



Cayman Scientific (2014)

Figure 3. Principle of CDNB colorimetric assay for GST. CDNB is a compound targeted by GST for conjugation with reduced glutathione. CDNB-glutathione conjunction corresponds with increased sample absorbance at 340 nm, which can be detected using a visible light spectrometer. Changes in sample absorbance at 340 nm is used to measure GST activity quantitatively.

Table 1. Conditions of assay. 6xHis-GST was purified via IMAC. Reduced glutathione (GSH) was dissolved in the 0.1 M potassium phosphate buffer, pH 6.5. CDNB was dissolved in 10% ethanol. Concentrations of GSH and CDNB are based on Provost & Wallert (2012). Buffer conditions were adapted from Cayman Scientific (2014).

Conditions of bi-substrate assay

Enzyme	6.2 mg/mL 6xHis-GST
Buffer	0.1 M Potassium Phosphate, pH 6.5
Substrate 1	100 mM GSH
Substrate 2	100 mM CDNB

Endpoint assay

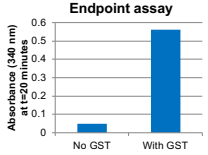


Figure 4. Endpoint assay validating the activity of GST. No GST: 20 µL of GSH and CDNB and 1960 µL of the buffer. With GST: 40 µL of GST, 20 µL of GSH, 60 µL of CDNB and 1880 µL of buffer (refer to Table 1).

Increasing [GSH] increases GST activity

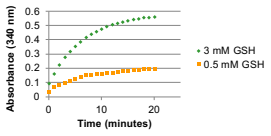
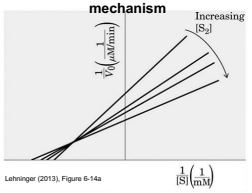


Figure 5. Representative reaction progress curves. Increased glutathione transferase activity with increasing GSH concentration is depicted.

Kinetic Models for Bi-Substrate Reactions

Michaelis-Menten model for sequential bi-substrate mechanism



Lehninger (2013), Figure 6-14a

Figure 6. Michaelis-Menten model for a random or ordered sequential bi-substrate catalysis. Intersecting lines exhibiting changes in K_m, V_{max}, and slope (K_m/V_{max}) indicate a sequential reaction mechanism.

Dead-end inhibition of GST

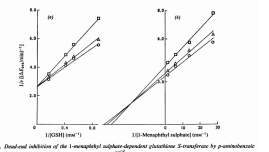


Fig. 5. *Dead-end inhibition of the 1-menaphthyl sulphate-dependent glutathione S-transferase by p-aminobenzoic acid*

The final concentrations of 1-menaphthyl sulphate (s) and GSH (t) were 0.17mM and 2.44mM respectively. The concentrations of *p*-aminobenzoic acid were: ○, none; △, 0.23mM; □, 0.58mM.

Gilham (1973)

Figure 7. Dead-end inhibition by *p*-aminobenzoic acid at varying concentrations of GST substrates. Inhibitor is competitive with GSH and non-competitive with 1-menaphthyl sulfate, indicating an ordered bi-substrate mechanism, with GSH binding first.

Future Directions

- Confirm whether GST mechanism is that of a sequential bi-substrate reaction by application of Michaelis-Menten model
- Use inhibitor profiles with the reported dead-end inhibitor, *p*-aminobenzoic acid in order to characterize the mechanism of the enzyme (ordered sequential over random sequential)
- Apply established assay methodology for investigation of GST kinetics in the introductory biochemistry lab

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