Rapid and Specific HILIC Method to Assay Guanine Deaminase and Application to Characterize the Brain Enzyme

Barry Boyes, William Miles, Benjamin Libert, and Stephanie Schuster
Advanced Materials Technology, Inc., Wilmington, DE 19810

Presented at ISPPP 2015, Philadelphia

Introduction

Guanine deaminase, also variously known as "nedasin " or "cypin", catalyzes the purine catabolic commitment step from guanine, through xanthine, to the elimination product, uric acid. In rabbit and human, the enzyme appears to exist predominantly cytoplasmic as a homodimer, with catalytic domains for the Zn^{+2}-dependent hydrolytic deamination of guanine to xanthine plus ammonia. For humans, genomic details for the GDA gene are well mapped, and expression profiling in certain tissues and organisms has been initiated.

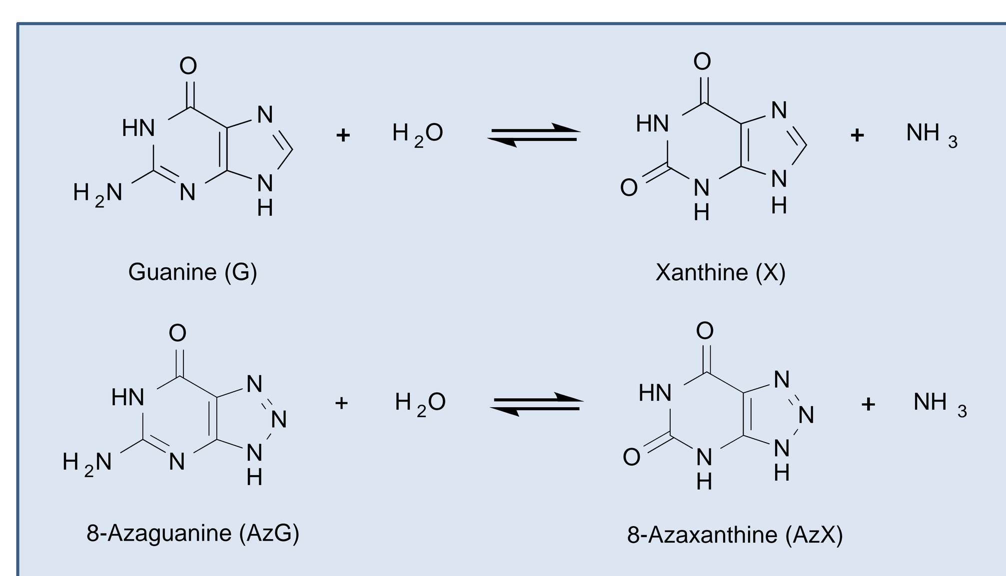
- Structure: c. 50 kDa subunits with sequences that vary at internal and terminal sites, due to exon selection; at least 4 significant forms of various chain lengths are known to exist.
- Interactions: tubulin, snapin, and the protein post-synaptic domain protein 95 (PSD-95).
- PSD-95 binding is through the PDZ binding motifs present at the C-terminus of the dimeric structure.
- Sequence variants occur mostly at the protein binding domains, although minor variants lack the deaminase catalytic site.
- In mammalian brain, high levels are in telencephalic brain regions; low levels in white matter and cerebellum.
- Moderate levels in liver and certain other specialized organs.
- Low levels in plasma/serum; altered by liver dysfunction.
- Actual role of guanine deaminase in specialized organ metabolism and synaptic physiology is uncertain, and relatively little is known about the enzyme characteristics outside of rabbit, with considerable more available detail on human gene expression patterns, but not at the protein level. Available protein expression surveys have suggested extensive post-translational modifications, that have not yet been detailed with certainty.

Purpose

- Previous tissue homogenate enzyme assays are complex, using coupled enzyme/cofactor indirect readout.
- Spectrophotometric high throughput assay has uncertain specificity and requires guanine as substrate near the limit of aqueous solution solubility, assays using NH_3 liberation, although useful for HTP, lack the specificity needed for tissue and fluid analysis.

To measure tissue enzyme levels, follow purification processes, and assay enzyme kinetics and inhibitors, LC/UV and LC/MS methods are needed. Our assay measures deamination of guanine to xanthine, using rapid HILIC separation. Crude homogenates use 8-azaguanine as substrate due to the presence of endogenous xanthine; product 8-azaxanthine reflects enzyme activity without correction for background. HTP assay and kinetic analysis with partially purified or purified enzyme measures the direct conversion of guanine to xanthine.

Reactions Catalyzed by Guanine Deaminase

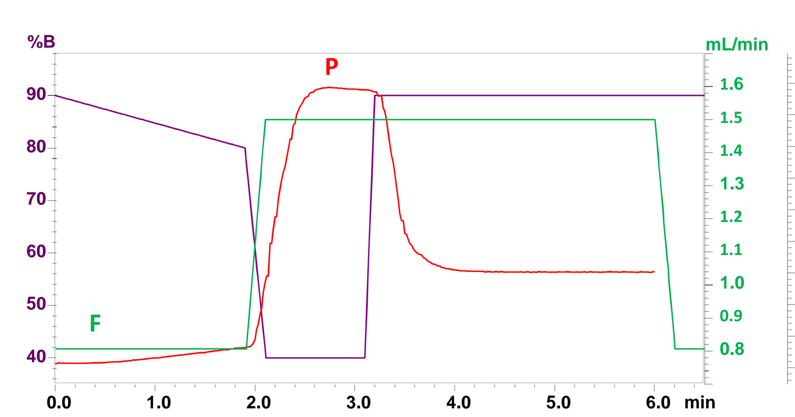


Rapid 2 µm HALO Penta-HILIC Analysis of Bovine Guanine Deaminase

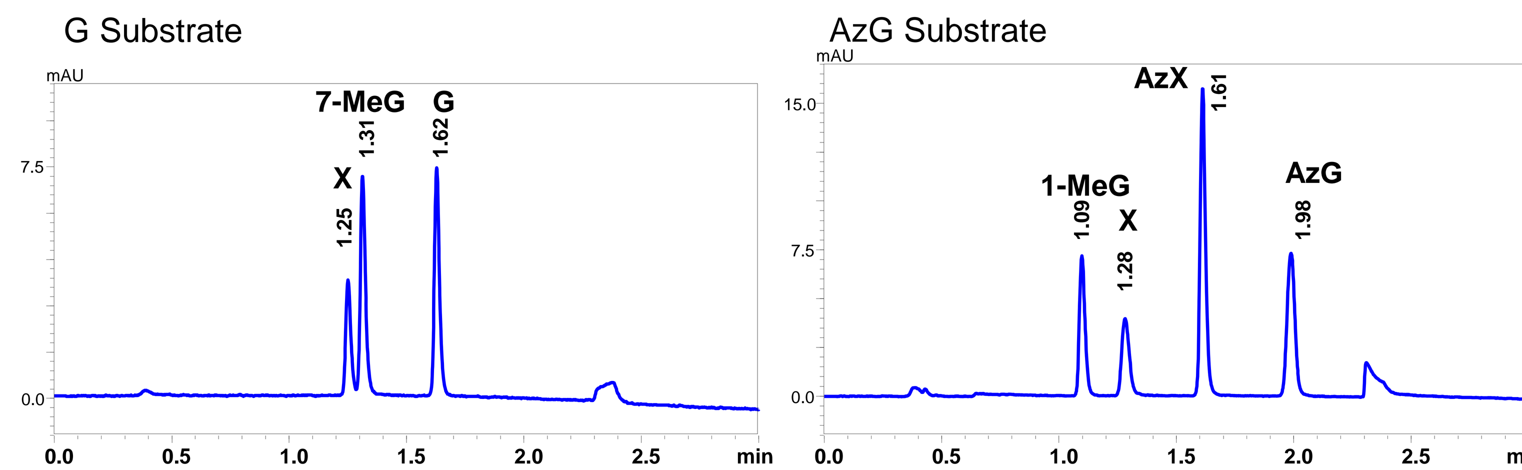
LC Conditions:

- 3.0 x 75 mm 2.0 µm particle HALO 2 Penta-HILIC
- Shimadzu Nexera with SPD-30A DAD at 270 nm (10 nm BW)
- 35 °C, 0.8 mL/min initial flow rate; 2-20 µL injection
- Eluents: A – 0.1 M NH_4OAc (pH 6.5); B – AcN
- For rapid equilibration, flow rate increased to 1.5 mL/min

Time	Component	Action	Parameter
0.00			
1.90	Pumps	Pump B Conc.	80
1.91	Pumps	Total Flow	0.8
2.10	Pumps	Total Flow	1.5
2.11	Pumps	Pump B Conc.	40
3.10	Pumps	Pump B Conc.	40
3.20	Pumps	Pump B Conc.	90
6.00	Pumps	Total Flow	1.5
6.20	Pumps	Total Flow	0.8
6.50	Controller	Stop	



Resolution of Standard Purines



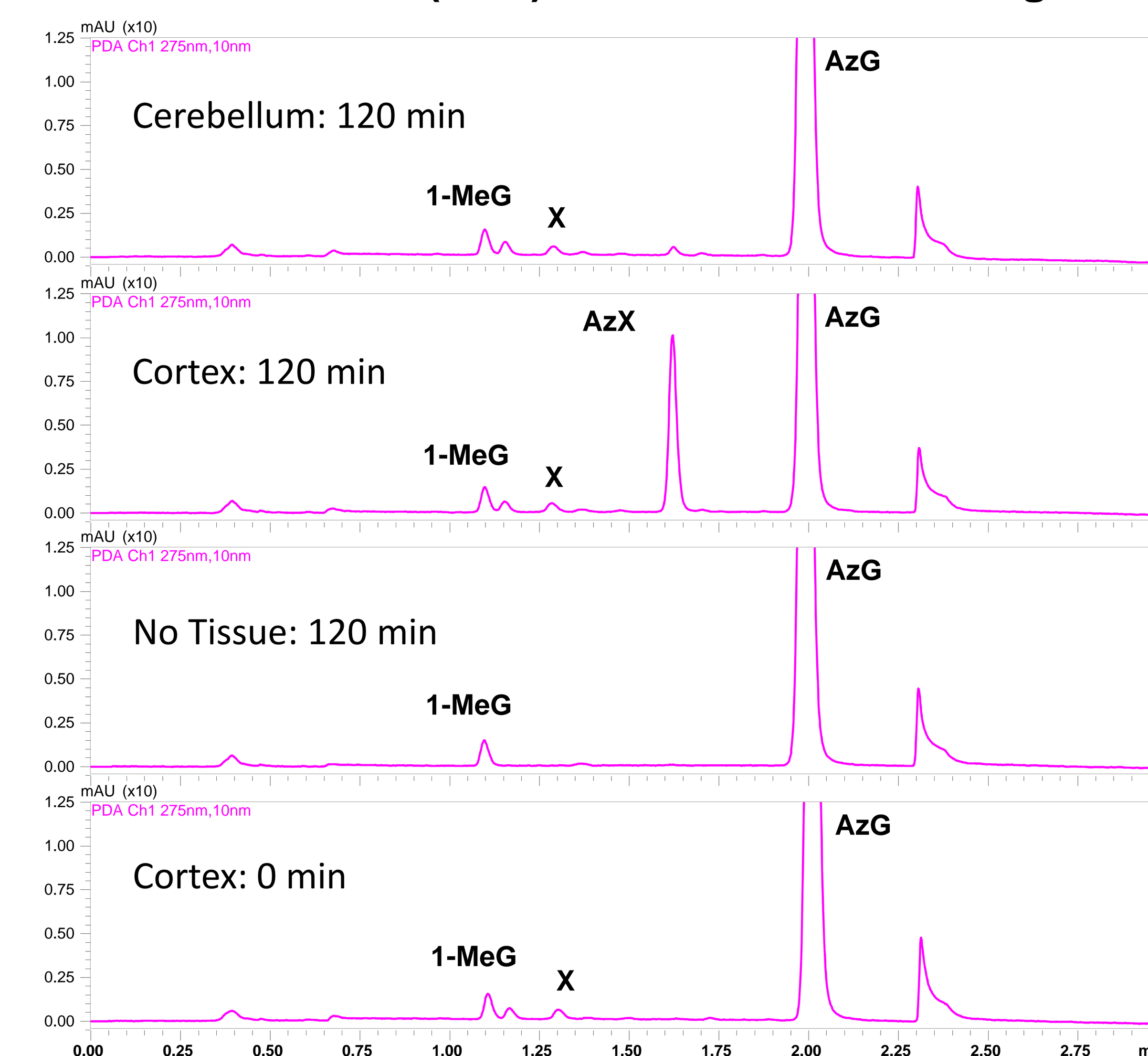
- Analysis time for this HILIC method is 6.5 minutes run to run, with complete re-equilibration of column
- Methyl-G can be used as an internal standard; 1-MeG has a preferred retention position
- Despite significant pressure changes, separation properties are highly reproducible.

Assay Conditions

Enzymatic Reaction:

- Temp: 25 °C
- Substrate: 0.2 mM G or 0.4 mM AzG
- Buffer: 0.1 M Bicine-HCl, pH 7.8
- Internal standard: 0.5 µM 1-methylguanine
- Stop Solution: 1% HOAc/99% Acetonitrile with IS
- Workflow:
  1. Incubate homogenate (10%) or purified enzyme in 10-100 µL of substrate mix
  2. 9 volumes Stop Solution, ice bath 5 minutes
  3. 10 minute centrifuge (16,000 x G)
  4. Direct injection of supernatant on LC

Guanine Deaminase (AzG) in Brain Tissue Homogenates

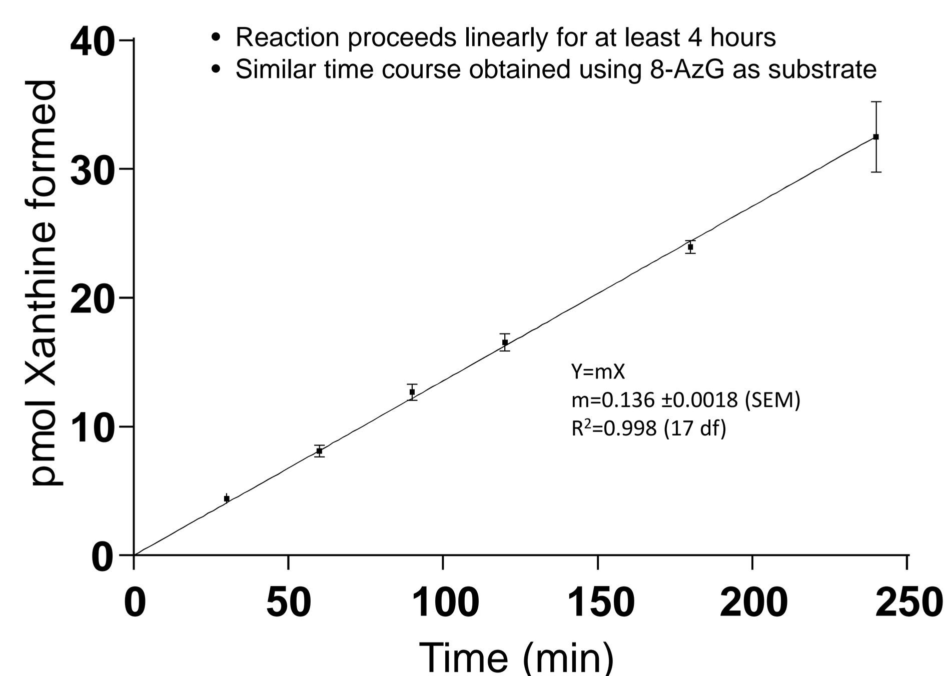


- In tissue homogenates, endogenous xanthine is a significant background for the enzyme assay
- Neocortex and cerebellum tissue homogenates samples exhibit endogenous X (and other unknown detected molecules)
- Use of 8-AzG as substrate yields 8-AzX as a product, well resolved from endogenous X and other compounds
- Neocortex shows 8-AzaX formed by guanine deaminase – no significant interferences from tissues at AzX or IS elution
- Cerebellum exhibits little guanine deaminase activity, as expected

Guanine Deamination is Stable for Extended Incubation Time

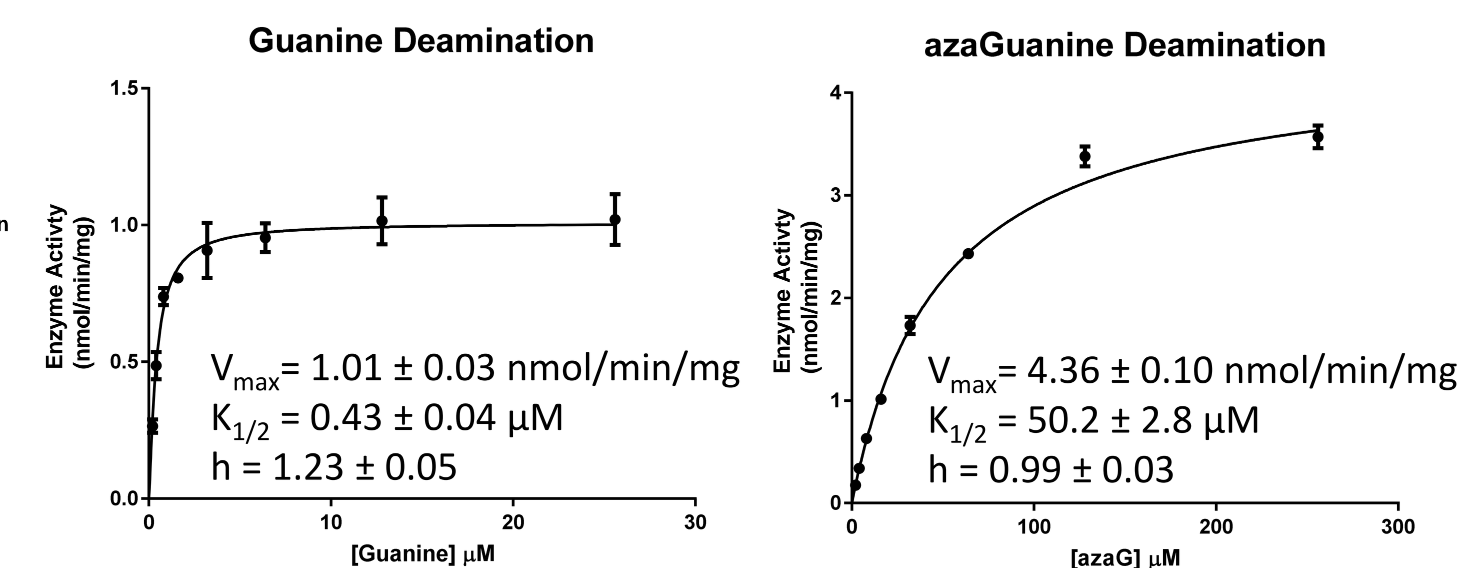
Guanine with Partially Purified Bovine Cortex Enzyme

25 °C; 0.1 M Bicine (pH 7.8); 200 µM Guaninine; 4 µg protein/reaction



Equilibrium Kinetic Analysis

- AzG appears to be a useful substrate for the enzyme assay, but initial results to compare activities using G and AzG yielded much higher AzX formation rates (c. 3-fold), than observed for X.
- Partially purified enzyme (80% saturated NH_4, followed by IEX, DEAE Sepharose FF; c. 80-fold enriched) was applied to study of substrate properties.



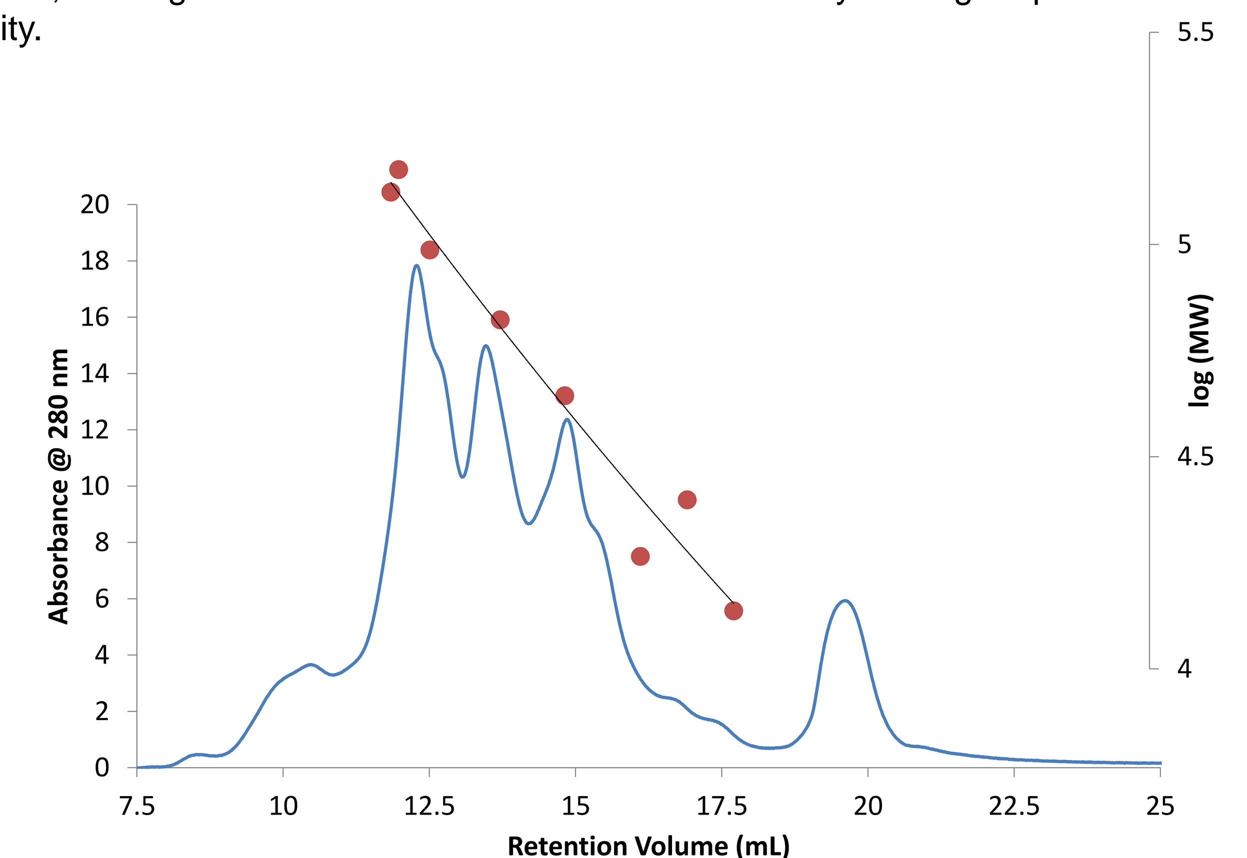
- Steady state reaction rates were fitted using non-linear least squares (GraphPad Prism), to the following rate equation:

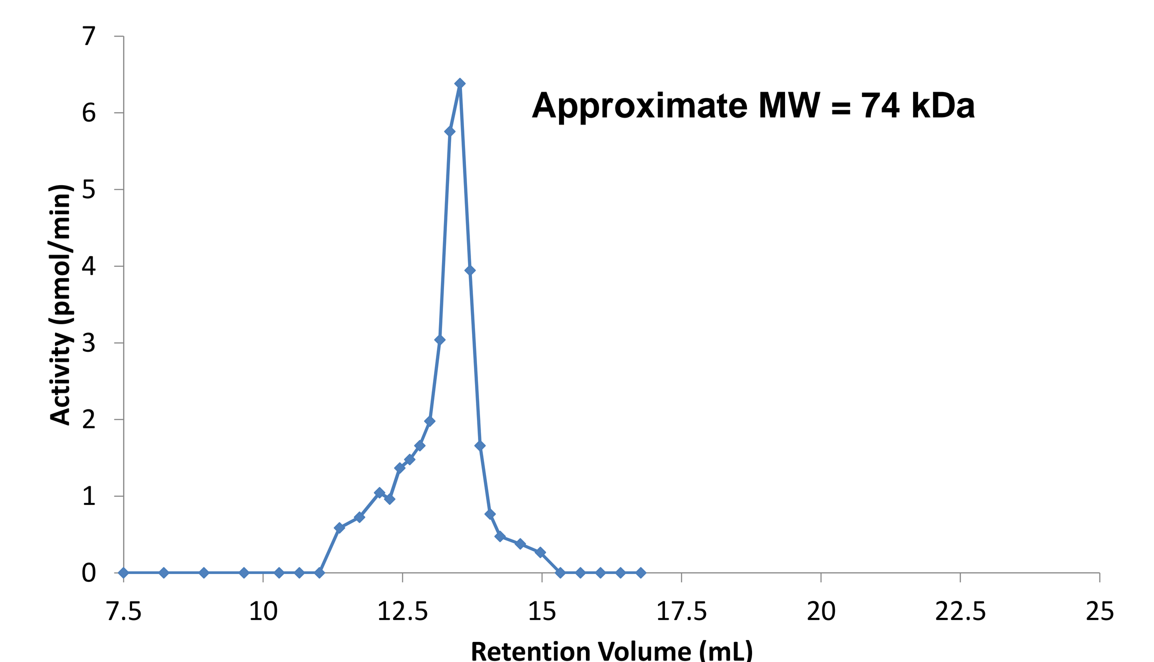
$$v = V_{max} * [S]^h / (K_{1/2}{}^h + [S]^h)$$

- V_{max} values for AzG were 4-fold higher than observed for G as substrate, and $K_{1/2}$ were also nearly 100-fold greater. The much greater V_{max} with AzG explains the much higher assay results obtained for tissue homogenates, compared to those using G as substrate.
- The h parameter for G was greater than 1.0, suggesting significant allosteric interactions for the (presumed) dimeric catalytic sites. Surprisingly, no allosteric effect was detected for AzG (h=0.99), which displayed nearly ideal Michaelis-Menton kinetic parameters (h=1.0).

Characterization of the Enzyme by Gel Exclusion Chromatography (SEC)

- Characterization of the partially purified enzyme by aqueous size exclusion chromatography was conducted to assess the apparent size and subunit structure of the bovine enzyme.
- Separation employed the GE Superdex 200 GL (10x300 mm) column, at 0.3 mL/min, using Tris-HCl buffered saline (TBS). Fractions were collected for activity assay using G with standard conditions.
- An apparent mass of 74 kDa was estimated, more consistent with a dimeric enzyme, although there was also observed some tendency for larger species of activity.





Conclusions and Future Directions

- A rapid HILIC assay, specific for guanine deaminase, is described. Current available methods are much less certain for specificity.
- Characteristics of the assay have been detailed, exhibiting kinetic properties unique to tested substrates. Enzyme measures require definition of the substrate employed.
- Comparing this work to the literature yields similar properties of the bovine brain, recombinant human and rabbit liver enzymes, as a dimer, but with specific kinetic differences from previous work.

Supported in part by NIH Grant GM093747 (BEB). Assistance from Bob Moran (AMT) is greatly appreciated.