Purification of Glutamate Dehydrogenase in Bovine (Bos taurus) Liver

Malika Prasad Ganguli

Abstract
Glutamate dehydrogenase. (GDH) is found in the mitochondrial matrix and catalyzes the reaction of L-glutamate to alpha-ketoglutarate, reducing NAD^+ to NADH in the process. Alpha-ketoglutarate is processed through the citric acid cycle and the electron transport chain, respectively, to produce the high-energy molecules NADH, $FADH_2$, GTP, and ATP. The purification of GDH has not been thoroughly explored in recent literature, thus driving the attempt in successfully isolating a kinetically active GDH from bovine (Bos taurus) liver. Bovine liver was homogenized in buffer and centrifuged to remove cell debris. Ammonium sulfate fractionations (ASF) removed unwanted proteins, and two samples were saved: 50% ASF and 70% ASF. Each sample underwent ion exchange (IEX) and size exclusion chromatography (SEC). Kinetic assays were used to analyze total catalytic activity in which the reverse reaction was monitored, where NADH consumption was measured at $340\,\mathrm{nm}$. In the 70% ASF sample, $17.00\,\mathrm{mg}$ of total protein remained after SEC and showed the greatest amount of NADH consumption, with an enzyme activity of $1.44 \times 10^{-10}\,\mathrm{U}$ and a specific GDH activity of $8.47 \times 10^{-12}\,\mathrm{U/mg}$ at $22\,^{\circ}\mathrm{C}$ and pH 8.0. The 50% ASF sample showed less NADH activity, with an enzyme activity of $1.05 \times 10^{-8}\,\mathrm{U}$, a specific GDH activity of $3.5 \times 10^{-10}\,\mathrm{U/mg}$, and a total protein mass of 30.00 mg at $22\,^{\circ}\mathrm{C}$ and pH 8.0. Quantifiable GDH identification on SDS-PAGE showed a dark band at the expected protein location, $\approx$ 60 kDa. Thus, this study provides an experimental procedure for the isolation of GDH from bovine liver with satisfactory efficacy.

Keywords
Metabolism — Mitochondrial enzymes – Enzyme purification

Key Words and Abbreviations

- ASF: Ammonium Sulfate Precipitation
- CAC: The Citric Acid Cycle
- DTT: Dithiothreitol
- $FADH_2$: Flavin Adenine Dinucleotide
- GDH: Glutamate Dehydrogenase
- GTP: Guanosine 5'- Triphosphate
- IEX: Ion-exchange Chromatography
- NADH: Nicotinamide Adenine Dinucleotide-Hydrogen
- PB: Phosphate buffer (0M NaCl)
- PBS: Phosphate Buffer (0.25M, 0.5M, 0.75M, and 1.0M NaCl)
- SDS-PAGE: Sodium dodecyl sulfate (C12H25SO4Na) Gel Electrophoresis
- SEC: Size Exclusion Chromatography
- TRIS: Tris(hyroxymethyl)aminomethane buffer

Contents

1. Introduction

In eukaryotic metabolism, intracellular enzymes coordinate the catalysis of reactions that produce and manage the function of essential biomolecules. Such biomolecules include high-energy molecules (i.e. adenosine triphosphate, ATP), proteins, and cell structures.

In mammalian liver, glutamate dehydrogenase (GDH) is found in the mitochondrial matrix and is a prominent enzyme used in metabolism to form ATP. GDH catalyzes the oxidation of the amino acid L-glutamate into alpha-ketoglutarate (α - ketoglutarate), an intermediate of the Citric Acid Cycle (CAC). Alpha-ketoglutarate is processed through CAC into CO_2, while facilitating the production of the high-energy molecules, reduced nicotinamide adenine dinucleotide- (NADH), reduced flavin adenine dinucleotide ($FADH_2$), and guanosine 5'- triphosphate (GTP). The energy payoff from NADH, $FADH_2$ are far greater than that of ATP, while GTP provides energy at similar levels to ATP. NADH and $FADH_2$ are further processed in the electron transport chain, along the mitochondrial inner membrane. Ultimately, GDH is essential in amino acid catabolism and the production of energy from the protein sources.

Mitochondrial GDH is coded by the gene GLUD1 (UniProtKB accession number P00366) (Weiss & Refetoff, 2015). GDH is a homohexamer, where each subunit is 61 512 Da and 558 amino acids long (Figure 1) (Weiss & Refetoff, 2015). The fully assembled protein is 360 072 Da. The isoelectric point of GDH is 7.25, and is slightly negatively charged throughout the experiment, as it is suspended in buffers (TRIS, PB, or PBS) at pH 8.0 (Weiss & Refetoff, 2015). The half-life of mammalian GDH is 14 hours (Li, Li, Allen, Stanley, & Smith, 2011). As a dehydrogenase, GDH reduces NAD+ into NADH. The nicotinamide cofactor binds between amino acids 141 to 143 on GDH. The activity of GDH is allosterically regulated by GTP, ADP, leucine, steroid hormones, and other ligands (Weiss & Refetoff, 2015;(Li et al., 2011)). The reaction scheme of glutamate dehydrogenase converts L-Glutamate to alpha-ketoglutarate uses NADH, and is stabilized by purine nucleotides (ADP) in solution ((McComb, Bond, Burnett, Keech, & Bowers, 1976).

$$\begin{aligned} &\text{L}-\text{Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \longrightarrow \\ &\quad \alpha\text{-ketoglutarate} + \text{NADH} + \text{NH}_4{}^+ \end{aligned} \tag{1}$$

The isolation of GDH has previously been explored through the late 1960s to the 1980s ((Di Prisco & Strecker, 1970; King & Frieden, 1970; McCarthy, Walker, & Tipton, 1980; McComb et al., 1976). The most recent literature exploring the isolation of bovine mitochondrial liver GDH was in 2015 (Eisenberg & Tomkins, 1968). The primary objective of this experiment is to isolate glutamate dehydrogenase from a sample of raw bovine liver and to explore the efficacy of isolation techniques from literature in preserving GDH enzyme activity.

Standard biochemistry procedures were used in this experiment, where disrupted liver tissue was fractionated through a series of centrifugation cycles. The removal of unwanted proteins from supernatant was carried out through ammonium sulfate precipitation. Literature shows GDH precipitation at 50% ammonium sulfate (ASF) and 60% ASF concentrations(McComb et al., 1976). To determine the efficiency of different ammonium sulfate concentrations, two treatments were explored in this project: a 50% ASF and a 70% ASF sample. Dialysis and chromatography removed ammonium sulfate from the filtrate. Ion Exchange (IEX) and Size Exclusion Chromatography (SEC) methods further removed unwanted proteins from the samples. The final treatments (post IEX, SEC treatments) were analyzed via kinetic traces, where reverse reaction of GDH measured NADH consumption and dehydrogenase activity (Chee, Dahl, & Fahien, 1979). Spectroscopically, NADH absorbance is measured at 340 nm. Total protein masses were measured by Bradford Assay (GDH + other proteins in solution), while the quantifiable GDH identification was carried out via SDS-PAGE; successful purification was indicated by the presence of a prominent band at 60kDa.

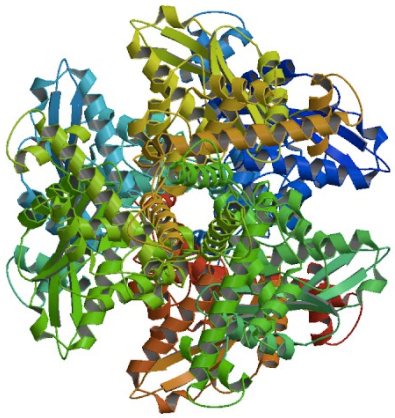

Figure 1. *Glutamate Dehydrogenase (GDH) 3-dimensional structure. The protein has a closed, cyclical structure with six subunits. The structure is primarily composed of alpha helices and looped regions*[1]

2. Procedure

All equipment for the purification procedure for Glutamate Dehydrogenase (GDH) was provided through the Trent University Chemistry Department. The half-life of bovine GDH is 14 hours, emphasizing the importance of storing the enzyme solution on ice, in the cold room, and in an ammonium sulfate salted pellet when possible (McComb et al., 1976).

2.1 Buffers

Stock solutions of 10mM Tris(hyroxymethyl)aminomethane (TRIS) buffer (with 0.5 mM EDTA, 1 mM PMSF) at pH 8.0 were used (Chee et al., 1979). TRIS was used for the majority

of the procedure, with the exception of Ion-exchange (IEX) and Size Exclusion Chromatography (SEC). Phosphate buffer (PB) with 0.5mM EDTA at pH 8.0 and Phosphate Buffer Saline (PBS) with 0.5 mM EDTA at 25 mM NaCl (pH 8.0) were also stored, for use in the chromatography (Chee et al., 1979). HCl and NaOH were used to adjust the pH of the buffers to pH 8.0. H^+, Cl^-, Na^+, and OH^- were spectator ions in solution and were assumed to have little impact on the stability of GDH. All solutions were kept on ice, or in the cold room at approximately 4 °C.

2.2 Preparation of tissues

Bovine GDH was purified from the Bos taurus liver. All connective tissue, fat, and unnecessary accessory tissue was removed from a 50 g sample of the liver sample, which was soaked in saline (0.15M NaCl) for 5 minutes to remove blood (Chee et al., 1979). The saline was drained, and approximately 100mL of TRIS buffer was added to the tissue, which was homogenized into a pulp using a Waring Blendor (Waring Laboratory Science, Stamford, CT, USA). The blender was turned on for 30 seconds, then paused for 30 seconds for cooling, and repeated until the tissue was homogenized to avoid protein denaturation from heat and frothing. The homogenate was strained through a cheesecloth to remove accessory and solid liver tissues and placed on ice. About 70mL of homogenate was collected after initial preparation.

2.3 Centrifugation

The homogenate was centrifuged multiple times to remove cell debris and insoluble tissue. The sample was spun in a Sorvall 6B Plus refrigerated centrifuge (Thermo-Fisher Scientific, Waltham, MA, USA) at 25,000g for 30 minutes at 4 °C (SS-34 rotor). The supernatant was extracted and recentrifuged at the same parameters as the first cycle (Chee et al., 1979). The supernatant was extracted and prepared for ammonium sulfate precipitation. The pellets were discarded.

2.4 Ammonium Sulfate Fractionation

Using dry ammonium sulfate powder, the supernatant from the second round of centrifugation was brought from 0% to 25% saturation, then centrifuged at 25 000 g for 30 minutes at 4 °C(SS-34 rotor) (Chee et al., 1979). The pellet was discarded. The supernatant was extracted, then brought from 25% to 50% saturation and centrifuged. The pellet of the 50% fraction was extracted and dissolved in 20mL of TRIS buffer. The supernatant of the centrifuged 50% fraction was brought from 50% to 70% saturation and centrifuged. The supernatant of the 70% fraction was discarded. The pellet of the 70% fraction was dissolved in 20mL of TRIS buffer.

Thus, two solutions were examined in the remainder of the project: 50% and at 70% ammonium sulfate concentrations. Going forward into the report, these will be referred to as the '50% fraction' and '70% fraction.' The two fractions were placed into dialysis tubing and dialyzed to desalt the pellets. The tubing was placed in 1 L of TRIS buffer. The buffer was replaced every 5 hours for a total of 4 repetitions. Molecular weight cut-off for the dialysis tubing is $<<<$ 360kDa.

2.5 Storage of samples

All samples were stored in the Trent University Cold Room at 4 °C, over the course of 10 weeks. Optimally, long-term storage of samples (over 2 weeks) should be in ammonium sulfate pellets, however, this was not carried out during storage due to time constraints.

2.6 Ion Exchange Chromatography (IEX)

To remove insoluble material that could otherwise clog the chromatography columns, samples were spun for 5 minutes in a tabletop microfuge and supernatant was separated from the pellet[9]. The supernatant of the 50% and 70% fractions were run through DEAE-Sepharose columns, equilibrated with Phosphate buffer (pH 8.0) (Chee et al., 1979). The columns were 6 cm tall x 1.5 cm diameter (50% ASF) and 5 cm tall x 1.5 cm (for 70% ASF).

The IEX column was developed under gravity flow. First, 2mL of the GDH sample was added into the column. 2mL of 0M NaCl Phosphate Buffer (loading buffer) was run through the column, collected, and labelled 'Fraction 0.' A stepwise gradient of increasing salt concentration (0.25 M, 0.5 M, 0.75 M, 1.0 M of NaCl in Phosphate Buffer) were added to the column to elute GDH (Chee et al., 1979). 2mL of each fraction were applied to the column, collected, and labelled as 'IEX Fraction 1' to 'IEX Fraction 5' with increasing NaCl concentration. An enzyme assay was conducted on each sample (see 2.10. Dehydrogenase Activity Measurement). It was determined that the 50% fraction had the highest dehydrogenase activity in IEX Fraction 5 (1.0 M PBS), while the 70% fraction had the highest dehydrogenase activity in IEX Fraction 4 (0.75 M PBS). These IEX fractions were used for the remainder of the experiment.

2.7 Size Exclusion Chromatography (SEC)

The 50% and 70% IEX fractions were run through a Size Exclusion Chromatography (SEC) column (22 cm tall x 1.5 cm wide) in Sephacryl S-200 resin (Chee et al., 1979). The column was equilibrated in phosphate buffer (0 M NaCl, pH 8.0). The SEC samples were developed by a pump (at speed 7, $\approx$1mL/minute flow rate). After equilibration, 1.6mL of the 50% and 70% IEX fractions (with the highest enzyme activity) were run through the column and collected into 5 fractions.

Fractions ranged from SEC Fraction 1 to 5. 12mL of 'SEC Fraction 1' was collected, with proteins larger than 400kDa. 'SEC Fraction 2' expected to have GDH with 10mL collected. Proteins in Fraction 2 are approximately 100-400kDa, with GDH at $\approx$360kDa. 5mL of 'SEC Fraction 3' was collected and contained proteins slightly smaller than GDH. 5mL of 'SEC Fraction 4' was collected and contained proteins considerably smaller than GDH. 5mL of 'SEC Fraction 5' was collected and primarily contained buffer and salts.

Before the sample was run through the column, a 2-mL sample of blue dextran (Sigma Aldrich, St. Louis, MO, USA) was run through as a marker, to determine the approximate volume of buffer that would pass through the column before GDH elutes. Approximately 13mL of PBS was eluted before blue dextran ran through the column (of which, 8.5mL was collected). Blue Dextran was clearly visible during chromatography (dyed dark blue, while the GDH solution is clear) and is approximately the same size (≈400kDa) as GDH (≈360kDa) and is an appropriate marker.

Five SEC fractions were collected: Fraction 1 (12mL, large proteins eluted before the enzyme), Fraction 2 (10mL, expected enzyme location), Fractions 3 and 4 (5mL, smaller proteins eluted after the enzyme), and Fraction 5 (5mL, primarily salts). GDH was expected to elute in fraction 2 (approximated from the Blue Dextran trial) and the enzyme kinetics of Fraction 2 were conducted (see 2.10. Dehydrogenase Activity Measurement).

2.8 Quantifiable GDH identification (SDS-PAGE)

Protein sizes were identified through SDS-PAGE. All samples were run for 90 minutes at 180V in 10% polyacrylamide gel. Dialyzed protein samples without undergoing chromatography were diluted (20-fold) with de-ionized water to avoid over-concentrating the lane on the gel. SEC protein samples were run through the gel undiluted, as the SEC naturally dilutes samples.

2.9 Protein mass quantification

The total protein masses were measured by Bradford Assay. Spectroscopically, the total protein mass (GDH + all other proteins) were measured.

2.10 Dehydrogenase Activity Measurement

Dehydrogenase enzymes use nicotinamide cofactors (NAD+ and NADH) to catalyze reactions. In glutamate dehydrogenase, NAD+ is a substrate in the forward reaction from L-glutamate into alpha-ketoglutarate, while the reverse reaction consumes NADH. NADH absorption at 340nm was measured on a Cary-50s and Cary-60s Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) (Chee et al., 1979). The molecular absorptivity of NADH is 6317 L/mol.cm (± 5.5 ppt) (Li et al., 2011). The enzyme assays were run at pH 8.0 and at room temperature, approximately 4 °C.

The ΔG for the reaction catalyzed by GDH is ≈0, thus the reverse reaction readily occurs and was carried out in this experiment (McCarthy et al., 1980). A stock solution of 150 μL of 0.1 M alpha-ketoglutarate, 150 μL of 1 M ammonium chloride, 20 μL of 18 mM adenosine diphosphate (ADP), 50 μM NADH, and 2500 μL of 10 mM TRIS buffer (0.5 mM EDTA, pH 8.0) was prepared (Chee et al., 1979). To test the dialyzed ammonium sulfate sample, 1900μL of this assay buffer and 10μL of GDH crude sample was mixed thoroughly, then the consumption of NADH was observed over a period of 10 minutes. A linear, decreasing absorbance at 340nm indicates NADH consumption catalyzed by GDH.

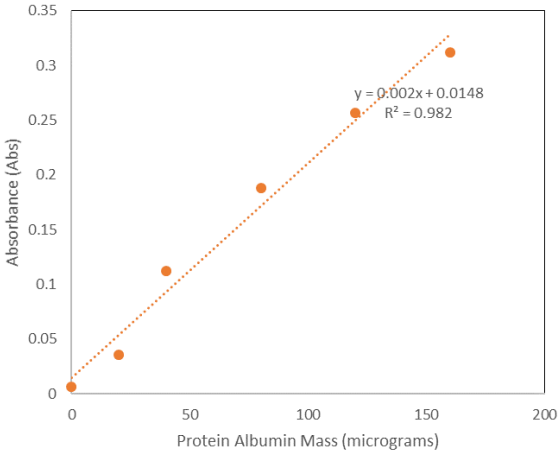

Figure 2. *Bradford Assay Standard Curves 1 and 2*

To test the SEC samples, 1950 μL of the stock solution and 50μL of the GDH sample was tested on the spectrophotometer, using the same parameters as above. It is important to note that all dehydrogenase proteins utilize NAD+ and NADH as cofactors. While the isolation of glutamate dehydrogenase was attempted, the solution may have contained other dehydrogenase proteins that consumed NADH. The spectrophotometer cannot measure the activity of GDH alone, and may measure the NADH consumption from other dehydrogenases.

3. Results

Glutamate dehydrogenase was successfully purified in this experiment. The success of this project was carried out by identifying the sizes of proteins within the solution on an SDS-PAGE gel (see 2.8. Quantifiable GDH identification), identifying the total protein mass in solution with a Bradford Assay (see 2.9. Protein Mass Identification), and NADH consumption on an enzyme assay (see 2.10. Dehydrogenase Activity Measurement).

3.1 Total Protein Mass

Total protein mass (GDH + other protein contents) was measured in the purified solutions via Bradford Assay. A standard curve, using known protein masses from bovine serum albumin, was created (Figure 1, Equation 2).

The average protein mass (from several trials) is summarized in Table 1 (See Appendix II for sample calculation). As expected, the largest protein masses were found in early samples, such as the ammonium sulfate (ASF) fractions prior to chromatography, with values well over 1500 mg. The 50% and 70% fractions collected from size exclusion chromatography (the last isolating technique used) contained the smallest protein masses at 30.00 and 17.00 mg respectively. These values indicate unwanted proteins removed during each isolating step.

$$y = 0.002x + 0.0148 \quad (2)$$

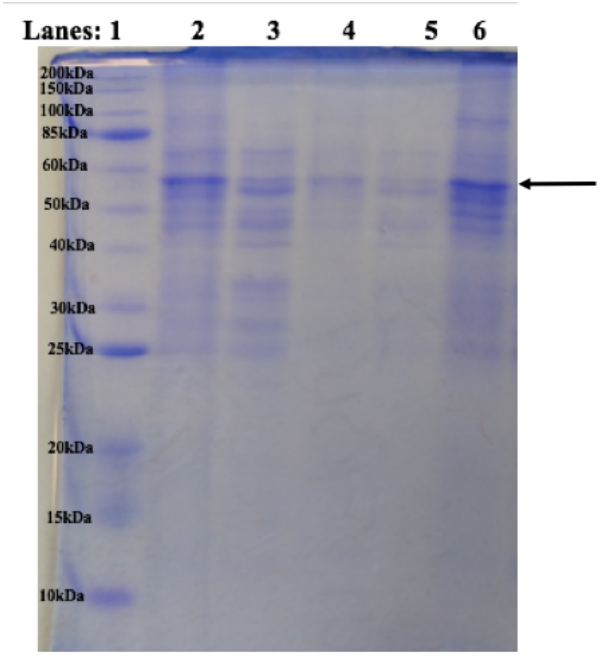

Figure 3. *SDS-PAGE Results. Lane 1 is the protein ladder. Lanes 2 shows the 50% IEX sample (1.0M PBS, NaCl), while Lane 3 shows the 70% IEX sample (0.75M PBS, NaCl). Lanes 4 and 5 show the 50% and 70% SEC samples, respectively. Lane 6 shows the 50% crude sample (dialyzed, after ammonium sulfate salting). The yellow arrow denotes GDH location amongst lanes 2-6.*

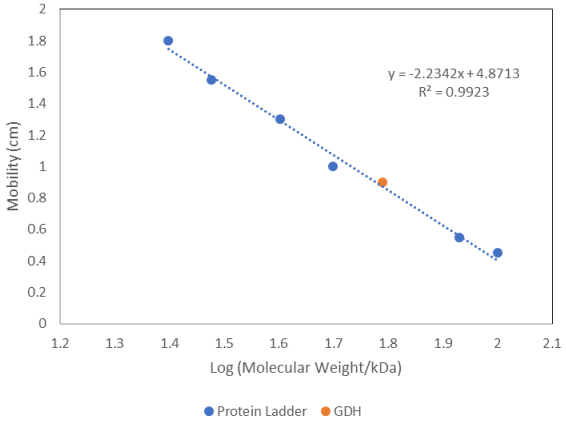

Figure 4. *Estimating the molecular weight of GDH (plotted in orange) through a standard curve of the protein ladder (blue). Extreme values were omitted to accommodate for the deviation of the trend line. Omitted values are summarized in Table 2.*

3.2 SDS-PAGE

Glutamate dehydrogenase is a homohexamer. The total protein size is 369 072 Da, with each subunit being 61 512Da and 558 amino acids long. The homohexamer denatures into individual subunits when mixed with 2X Sample loading buffer. Thus, a band at approximately 60 000Da suggests GDH presence. Many early gels showed a **highly-concentrated** protein sample and were diluted in following runs of SDS-PAGE, as the bands leaked into neighbouring lanes.

The crude 50% and 70% samples (dialyzed after ASF, prior to chromatography) underwent a 20-fold dilution. The IEX chromatography samples underwent a 10-fold dilution. The SEC samples were not diluted. Lanes 2 to 6 show a side-by-side comparison; however, the 70% crude sample was not run in Lane 7 due to the lack of wells available (Figure 4). Lane 2 shows the 50% IEX sample (1.0M PBS, NaCl), while Lane 3 shows the 70% IEX sample (0.75M PBS, NaCl). Lanes 4 and 5 show the 50% and 70% SEC samples, respectively. Lane 6 shows the 50% crude sample (dialyzed, after ammonium sulfate salting). The darkest band in the red oval is the expected location of GDH.

The molecular weight of GDH was estimated using the results from Figure 4. The protein ladder in Lane 1 was compared to Figure 3, and plotted (Figure 6). The line of best fit from Figure 5 (Equation 3) was used to find the estimated size of GDH (Appendix III). Some data points on the upper and lower extremes were omitted (Table 2, Appendix III). Using this equation, the molecular weight of the band was calculated to be 60.29 kDa. The true molecular weight of GDH is 61.51 kDa (Chee et al., 1979; Prisco & Garofano, 1975; Wang, Fu, Wan, & Tang, 2015).

$$y = -2.2342x + 4.8713 \tag{3}$$

3.3 Measured Dehydrogenase Activity

Spectroscopic kinetic assays were conducted after each purification to determine GDH and other dehydrogenase activity by observing NADH consumption at 340nm. A steady, negative slope on dehydrogenase activity showed good activity.

After Size Exclusion Chromatography (SEC), the NADH absorption was measured in the 70% and the 50% ASF samples (Figure 6). The dehydrogenase activity was far more efficient in the 70% sample, with a clear, distinct, negative trend line (slope= -0.0432 Abs units/min). In comparison, the 50% sample did have some dehydrogenase activity (slope = -0.0008 Abs units/min), though may not be due to GDH (Figure 7). The differences in activity indicate a higher GDH concentration in the 70% fraction.

3.4 Specific Activity

The extinction coefficient of glutamate dehydrogenase is 58.79 x 106mM-1cm-1 (Bhagavan & Ha, 2011). Using the Beer-Lambert law (Equation 4), the specific activity of GDH can be found (Appendix IV).

$$\frac{d[substrate]}{dt} = \frac{\frac{da}{dt}}{\beta l} \tag{4}$$

Where $\frac{da}{dt}$ is the rate of change of absorbance, β is the extinction coefficient and l is the cuvette path length.

The specific activity of the 70% ASF fraction after SEC is 8.47×10^{-9} U/mg, while the 50% ASF fraction after SEC had a specific activity of 1.05×10^{-10} U/mg at 22 °C and pH 8.0

Table 1. *Average Protein Masses (mg), Calculated Spectroscopically (via Bradford assay)*

Sample	Dilution Factor	Average Protein Mass (mg)	Average Absorbance (Abs)
50% before IEX	100-fold	2326.25	0.10
70% before IEX	50-fold	1719.36	0.15
50% after IEX, at 0.75 M PBS	10-fold	581.75	0.25
70% after IEX, at 0.75 M PBS	10-fold	344.86	0.15
50% after IEX, at 1.00 M PBS	10-fold	125.33	0.26
70% after IEX, at 1.00 M PBS	No dilution	81.90	0.31
50% after SEC	No dilution	30.00	0.12
70% after SEC	No dilution	17.00	0.09

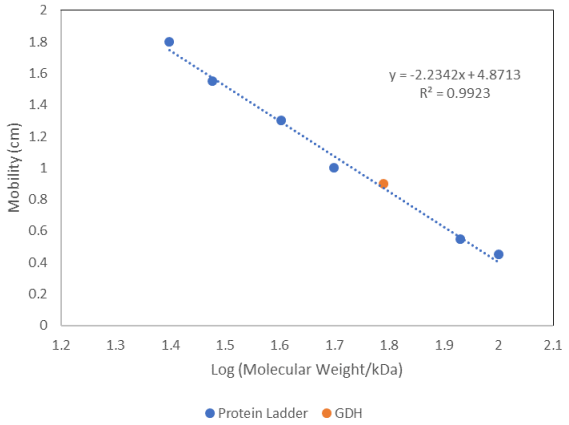

Figure 5. *Comparison of NADH consumption between SEC fractions of 50% and 70% ASF of GDH. The enzyme assays were run at pH 8.0 and at room temperature, approximately* 22 °C.

4. Discussion

In the spectral Bradford assay analysis, the protein masses decreased during each purification step. High protein masses were found in the samples prior to any chromatography, at 2326.25mg and 1719.36mg in the 50% and 70% samples, respectively. After size exclusion chromatography, the protein masses reduced to 30.00mg and 17.00mg for 50% and 70% samples. The drastic differences in protein masses indicate the removal of unwanted proteins in each chromatography step. The rate of NADH consumption was far greater in the 70% sample, indicating a greater dehydrogenase activity in the 70% sample. The 50% sample contained some dehydrogenase activity, though GDH is assumed to be present in the 70% sample. SDS-PAGE results show GDH presence in the 50% and 70% samples, with a dark band occurring at ≈60kDa in both lanes. Differences in the banding patterns between the 50% and 70% sample may be indicative of different proteins that salted out at the 50% and 70% ammonium sulfate concentrations. The lower of the two bands at ≈60kDa may be GDH (Figure 4).

Thus, based on the results of the Bradford assay, kinetic traces, and SDS-PAGE, there is evidence of a successful Glutamate Dehydrogenase Purification, with greater dehydrogenase activity in the 70% sample.

Some major concerns may have compromised the results of this study, and the purification of glutamate dehydrogenase. During spectral analysis, all samples contained a light pink hue, likely stained from residual hemoglobin. During ammonium sulfate centrifuge cycles, many of the unwanted proteins (including hemoglobin) were removed, though the heme proteins may have been broken down during the homogenization phase and leeched a red colour into the buffer. Spectroscopically, the red colour may alter the absorbance recordings. To obtain a clear colour, the liver tissue was soaked in a 0.15 M saline solution for 5 minutes. Some blood and red/pink colour was removed in this process, though more may have been removed if soaked for longer. Perhaps draining, and changing the saline solution once or twice may benefit the removal of the red colour. The addition of 250 μL of ethanol and 50 μL of chloroform has shown to be effective in removing hemoglobin remnants in superoxide dismutase (SOD) (Oztürk-Urek & Tarhan, 2001). Perhaps, the addition of ethanol and chloroform may remove the red hue for GDH. Other issues with kinetic traces are related to the homogeneity of the enzyme solution in the UV-VIS cuvette. A solution of 1950 μL of stock solution with 50 μL of GDH purified extract after IEX (see 2.10. Dehydrogenase Activity Measurement) for 10 minutes showed a non-linear line of NADH consumption, indicating improper mixing ("streaking" enzyme solution in the cuvette) and a high enzyme concentration in the solution. A reduction in IEX enzyme extract to 10μL GDH in 1990μL showed a linear, clear, and slow NADH consumption. Kinetic traces recorded for GDH samples after size exclusion chromatography used 1950μL of stock solution and 50μL of GDH. Size exclusion naturally dilutes fractions. All kinetic assays showed clear, linear, and slow consumption of NADH (Figure 6). Dehydrogenases (other than GDH) may consume NADH and interfere in the measurement of GDH activity. The addition of a control sample with ammonium chloride, ADP, NADH, TRIS buffer (pH 8.0) and GDH protein extract without the addition of alpha-ketoglutarate will indicate non-target dehydrogenase activity (see 2.10. Dehydrogenase Activity Measurement).

Protein stability was of major concern during the duration of this project. The experimenters were limited by the time commitment per week, as access to equipment was restricted to a maximum of 6 hours per week. As such, the project was carried out over a period of 10 weeks. The half-life of

mammalian glutamate dehydrogenase is 14 hours (McComb et al., 1976). All samples were placed on ice to minimize protein denaturation from heat exposure. All homogenization was conducted in 30-second intervals, with 30-seconds of break to reduce protein degradation from heat, movement, and excessive frothing. NADH addition may reduce protein stability. Purine nucleotides (especially adenine diphosphate, ADP) have shown evidence of maintaining protein stability in the presence of NADH (King & Frieden, 1970; McComb et al., 1976). ADP was added to GDH samples during the measurement of kinetic dehydrogenase activity, and successfully stabilized dehydrogenase proteins. Perhaps, the addition of ADP in buffer solutions may stabilize GDH during storage. GTP may be used as a purine substitute to ADP (King & Frieden, 1970). All samples were stored in a 4 °Ccold room, though the small half-life of the protein urges for the addition of other stabilizing factors. All buffers had 0.5mM of EDTA to protect against protease degradation. Dithiothreitol (DTT) could have been added to buffers to protect against oxidation and di-sulfide bond formation (Eisenberg & Tomkins, 1968). The addition of 20 or 30% glycerol may further stabilize the enzyme during storage (King & Frieden, 1970). It is not recommended to add glycerol to the spectroscopic stock solution during kinetic trace measurement as it may cause deviations in absorbance (McComb et al., 1976).

During ion exchange chromatography, the GDH underwent a buffer exchange from TRIS (pH 8.0) to Phosphate Buffer (PB) and Phosphate Buffer Saline (PBS) at pH 8.0 through DEAE-Sepharose resin. DEAE-Sepharose is an anion-exchange resin and binds to negatively charged molecules. The isolelectric point (pI) of GDH is 7.25 units, while the pH of the buffer solution (TRIS, PB, and PBS) were calibrated to pH 8.0. The pH of the solution was 0.75 units higher than the pI, thus, GDH is slightly negatively charged and will bind to the resin due to the deprotonation of the backbone carboxylic acid and amine groups, and the R groups of the amino acids. The buffer change may cause protein denaturation. In retrospect, the protein could have been eluted with TRIS buffer (pH 8.0) with increasing salt concentration (0M, 0.25M, 0.5M, 0.75M, 1.0M). DEAE-Sephadex A-25-120 could have been used as an alternative column (McComb et al., 1976)

Concerns with SDS-PAGE involved the salt saturation of samples and enzyme concentrations in each lane. Highly concentrated samples ran through the SDS-PAGE leaked into neighbouring lanes and did not show distinctive bands at the 60kDa region (expected size marker for GDH single subunit). A 20-fold dilution of samples with distilled water drastically improved the quality of SDS-PAGE results and showed clear bands in each lane. A dark band was found at ≈60kDa (Figure 4) indicating GDH presence. SDS-PAGE lanes showed bands from 25kDa to 200kDa, indicating poor separation from proteins of this size. During size exclusion chromatography, 10mL fractions were collected in the expected location of GDH protein. This fraction was far too large, as it collected proteins between the sizes of 25kDa to 200kDa, shown by the lighter bands on the gel (Figure 4). A second run through a size exclusion column (in Sephacryl S-200 resin) is recommended to produce a single band at 60kDa to isolate GDH. Fractions from the second round of SEC should be collected in 0.5mL increments to narrow the size band in each fraction. The high size range collected in the SEC fraction in this experiment may have compromised the results of kinetic traces. Other dehydrogenases between 25kDa and 200kDa may have converted NADH to NAD+ in the spectrophotometer and caused a linear NADH consumption. An ammonium sulfate fraction of 60% salt may be explored in further testing, reducing the range of protein sizes found on the SDS-PAGE gel (Figure 4) (McComb et al., 1976).

Enzyme activity defines the enzyme concentration that consumes 1 μmol of NADH/minute (i.e. total dehydrogenase concentration) (McComb et al., 1976). Specific activity defines the enzyme purity of GDH in solution. The 70% ammonium sulfate trails had a significantly better NADH consumption, with an enzyme activity of 1.44×10^{-10} U, while the 50% ammonium sulfate trial enzyme activity at 1.05×10^{-8} U (Appendix IV). The specific activity of GDH was calculated to be 3.50×10^{-10} U/mg and 8.47×10^{-12} U/mg for the 50% and 70% at 22 °C and pH 8.0 and after SEC. Literature records higher values of GDH specific activity, at 24.2 U/mg in King and Frieden, 40 U/mg in McCarthy et al., 80 U/mg in McDaniel et al., and 305.06 U/mg in Wang et al. (Eisenberg & Tomkins, 1968; King & Frieden, 1970; McComb et al., 1976). Specific activity is dependent on pH and temperature of the buffer. The optimal temperature for GDH activity was shown at 500C, however temperature is not controlled in the room. The use of a pH 8.2 TRIS or PB/PBS buffer may increase GDH activity (Eisenberg & Tomkins, 1968; McDaniel, Bosing-Schneider, Jenkins, Rasched, & Sund, 1986). It is possible the activity measurements of GDH is not limited to the liver isozyme. Mammalian GDH is found in the nuclei and mitochondria of rats and Chang's liver cells (King & Frieden, 1970; McDaniel et al., 1986). Current literature does not report the finding of bovine nuclear GDH, however, it may exist and consume NADH in kinetic assays. Isozymes may be differentiated by molecular weight on the SDS-PAGE. Furthermore, bicarbonate addition is correlated with increased mitochondrial activity of mammalian rat liver GDH and may be explored for bovine mitochondrial liver GDH (Merk et al., 2016; Oztürk-Urek & Tarhan, 2001).

In conclusion, the purification of glutamate dehydrogenase was successful. When comparing the two treatments, the 70% treatment showed better NADH consumption and protein activity, although the 50% treatment had a higher protein mass (Table 1, Appendix III). It is suggested that these two treatments are tested again, controlling for the red/pink hue from blood staining, addition of stabilizing reagents such as DTT and glycerol, avoiding a buffer exchange, using different buffer pH values (≈pH 8.2), controlling for reaction temperature, and narrowing the size range on the SDS-PAGE.

5. References

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6. Appendices

6.1 Amino Acid Sequence of GDH

The additional materials for this paper are included at trentu.ca/just.