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Work Towards the Isolation and Characterization of the Muscle Isoform of Glucose 1,6-Bisphosphatase

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Master of Science

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Abstract

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Glucose 1,6-bisphosphate is an important small molecule involved in the regulation of glycolysis. Four enzymes synthesize this compound. One enzyme is known to degrade it, *glucose 1,6-bisphosphatase*. Other groups have produced work that indicates that there are two isoforms of this enzyme, one predominant in the brain and one in the muscle. This thesis contains the work performed in attempts to isolate and characterize the muscle isoform of *glucose 1,6-bisphosphatase*. While this enzyme was not isolated, much was learned about it and the results from this work may help in the future identification of this enzyme.

Key words: *glucose 1,6-bisphosphatase*, glucose 1,6-bisphosphate, glycolysis

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Abbreviations

1,3-BPG	1,3-bisphosphoglycerate
3-PG	3-phosphoglycerate
Frc	fructose
Frc-1P	fructose 1-phosphate
Frc-6P	fructose 6-phosphate
Frc-1,6P2	fructose 1,6-bisphosphate
Frc-2,6P2	fructose 2,6-bisphosphate
Glc	glucose
Glc-1P	glucose 1-phosphate
Glc-6P	glucose 6-phosphate
Glc-1,6P2	glucose 1,6-bisphosphate
PMM1	*phosphomannomutase 1*

Equations

Equation 1 – reaction catalyzed by *glucose 1-phosphate transphosphorylase (1)* (E.C. 2.7.1.41)

$$2\ \text{Glc-1P} \rightarrow \text{Glc-1,6P2} + \text{Glc}$$

Equation 2 – reaction catalyzed by *glucose 1-phosphate kinase (2)* (E.C. 2.7.1.10)

$$\text{Glc-1P} + \text{ATP} \rightarrow \text{Glc-1,6P2} + \text{ADP}$$

Equation 3 – reaction catalyzed by *glucose 1,6-bisphosphate synthase (3)* (E.C. 2.7.1.106)

$$\text{1,3-BPG} + \text{Glc-1P} \rightarrow \text{3-PG} + \text{Glc-1,6P2}$$

Equation 4 – reaction catalyzed by *fructose 1,6-bisphosphate-dependent glucose 1,6-bisphosphate synthase (4)* (E.C. has not been assigned)

$$\text{Frc-1,6P2} + \text{Glc-6P} \rightarrow \text{Glc-1,6P2} + \text{Frc-6P}$$

Equation 5 – reaction catalyzed by *glucose 1,6-bisphosphatase (5)* (E.C. has not been assigned)

$$\text{Glc-1,6P2} + H_2O \rightarrow \text{Glc-1P} + P_i$$

Equation 6 – reaction catalyzed by *phosphoglucomutase*

$$\text{Glc-1P} \rightarrow \text{Glc-6P}$$

Equation 7 – reaction catalyzed by *glucose 6-phosphate dehydrogenase*

$$\text{Glc-6P} + NADP^+ \rightarrow \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADPH}$$

Chapter 1

Introduction

Glycolysis needs little introduction. It is one of the most studied enzymatic pathways in biochemistry, is central to metabolism, and is at the heart of metabolic flux. Nearly all organisms use this pathway to convert glucose to pyruvate via ten intermediate compounds.

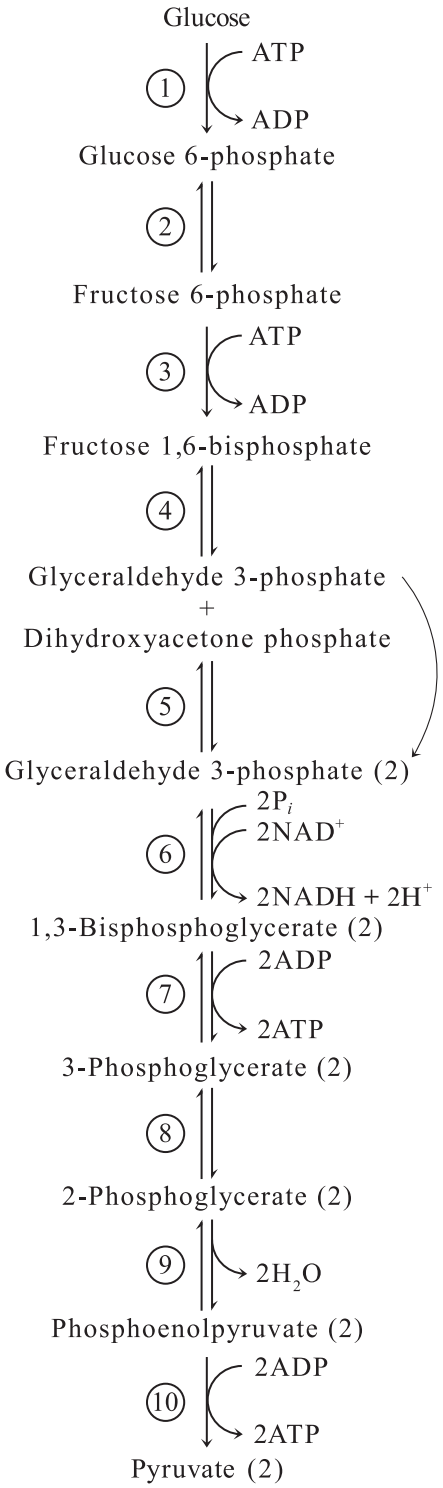
Glycolysis is commonly known to be carried out by the ten enzymatic reactions illustrated in Figure 1; however, it is actually more complex than illustrated in this figure.

Glycolysis is tightly intertwined with other metabolic pathways. As such, glycolytic intermediates are formed via other metabolic pathways and can be fed into and out of this pathway depending on the needs of the cell. The cell can thereby use excess glucose for long and short-term storage and the formation of amino acids.

Because glycolysis is so central to metabolism and proper cellular function, it is very tightly regulated. Flux through this pathway is controlled so that adequate amounts of glucose can be stored as glycogen/starch to be used later while maintaining a proper balance such that the energy needs of the cell can be met almost instantaneously.

The most well known regulator of glycolysis is fructose 2,6-bisphosphate, Frc-2,6P2. This compound receives a lot of attention because it is the most potent activator of *phosphofructokinase*, the rate-limiting enzyme of glycolysis. Glucose 1,6-bisphosphate, Glc-1,6P2, is also a potent activator of *phosphofructokinase*, but has not received the same acclaim. Our work has been focused on increasing the understanding of Glc-1,6P2.

Figure 1. Glycolysis.



	Enzyme
1.	*hexokinase*
2.	*phosphohexose isomerase*
3.	*phosphofructokinase*
4.	*aldolase*
5.	*triose phosphate isomerase*
6.	*glyceraldehyde 3-phosphate dehydrogenase*
7.	*phosphoglycerate kinase*
8.	*phosphoglycerate mutase*
9.	*enolase*
10.	*pyruvate kinase*

Glc-1,6P2 is a known regulatory compound for several metabolic enzymes, both glycolytic and non-glycolytic. A comprehensive list of the enzymes known to be regulated by Glc-1,6P2 is given as Table 1. The length of this list implies that the regulatory role of Glc-1,6P2 may not have a minor role in metabolic regulation. A figure illustrating the intricacies involving the role of Glc-1,6P2 in glycolysis will be given later. Before that figure can be given, background information on Glc-1,6P2 and the enzymes that regulate its formation and degradation is required for an understanding of the figure.

Table 1. Enzymes Activated and Inhibited by Glc-1,6P2.

EC #	Enzyme name	Glc-1,6P2	reference
1.1.1.44	6-phosphogluconate dehydrogenase	inhibits	*(6)*
2.7.1.1	hexokinase	inhibits	*(7)*
2.7.1.106	glucose-1,6-bisphosphate synthase	inhibits	*(8)*
3.1.3.11	fructose-1,6-bisphosphatase	inhibits	*(9)*
3.1.3.56	inositol-1,4,5-trisphosphate 5-phosphatase	inhibits	*(10)*
1.1.1.27	l-lactate dehydrogenase	activates	*(11)*
2.7.1.11	6-phosphofructokinase	activates	*(12)*
2.7.1.40	pyruvate kinase	activates	*(13)*
2.7.1.90	diphosphate-fructose-6-phosphate 1-phosphotransferase	activates	*(14)*
2.7.2.1	acetate kinase	activates	*(15)*
2.7.7.27	glucose-1-phosphate adenylyltransferase	activates	*(16)*
5.4.2.2	phosphoglucomutase	activates	*(17)*
5.4.2.3	phosphoacetylglucosamine mutase	activates	*(18)*
5.4.2.6	beta-phosphoglucomutase	activates	*(19)*
5.4.2.7	phosphopentomutase	activates	*(20)*
5.4.2.8	phosphomannomutase	activates	*(21)*
5.4.2.10	phosphoglucosamine mutase	activates	*(22)*

Glc-1,6P2 is not known to have any metabolic function other than to serve as a regulatory molecule. It is not metabolized. As such cellular concentrations of Glc-1,6P2 concentrations are tightly controlled. Passoneau et al. *(4)* showed that Glc-1,6P2 concentrations vary depending on the type of tissue, as shown in Table 2.

Table 2. Glc-1,6P2 Concentrations and Rates of Formation in Varying Tissues. Adapted from Passoneau et al. *(4)*.

Tissue	G16P2 (μmol kg^{-1})	G16P2 formation rate (mmol kg^{-1} hr^{-1})
red blood cells	80 ± 7	na
brain	72 ± 5	110 ± 8
spleen	57 ± 3	31.5 ± 2.4
muscle	45 ± 6	213 ± 12
muscle, stimulated	40 ± 4	na
lung	33.9 ± 3.4	16.5 ± 1.0
liver, starved	15.4 ± 1.5	na
kidney	14.4 ± 0.8	3.6 ± 0.3
liver	14.1 ± 0.8	1.46 ± 0.17
heart	12.2 ± 1.3	145 ± 12
pancreas	9.9 ± 1.5	na
testes	7.2 ± 0.4	20.5 ± 1.9

Four enzymatic reactions have been shown to synthesize Glc-1,6P2. These reactions are depicted as Equations 1-4. The corresponding enzyme name, E.C. number, and referenced citation follows:

Equation 1 – reaction catalyzed by *glucose 1-phosphate transphosphorylase (1)* (E.C. 2.7.1.41)

2 Glc-1P → Glc-1,6P2 + Glc

Equation 2 – reaction catalyzed by *glucose 1-phosphate kinase (2)* (E.C. 2.7.1.10)

Glc-1P + ATP → Glc-1,6P2 + ADP

Equation 3 – reaction catalyzed by *glucose 1,6-bisphosphate synthase (3)* (E.C. 2.7.1.106)

1,3-BPG + Glc-1P → 3-PG + Glc-1,6P2

Equation 4 – reaction catalyzed by *fructose 1,6-bisphosphate-dependent glucose 1,6-bisphosphate synthase (4)* (E.C. has not been assigned)

Frc-1,6P2 + Glc-6P → Glc-1,6P2 + Frc-6P

The enzymes responsible for catalyzing these reactions are under scrutiny. Each of these reactions has been classified as being a side reaction of another well-characterized enzyme. However, whether they are side reactions of other enzymes or are separate enzymes, these four reactions have been observed to form Glc-1,6P2. Several metabolic intermediates are used by

these enzymes to synthesize Glc-1,6P2, thereby deeply entrenching the role of Glc-1,6P2 with glycolysis.

The activity of the four enzymes that synthesize Glc-1,6P2 is different for each enzyme in various tissues. Climent et al. *(23)* reported the enzymatic activities of these four enzymes in various types of tissue, Table 3.

Table 3. Enzymatic Activities of Enzymes that Synthesize Glc-1,6P2. Adapted from Climent et al. *(23)*.

	Tissue (mU/g)			
	Skeletal Muscle	Heart	Brain	Liver
glucose 1-phosphate transphosphorylase	0.41 ± 0.07	0.53 ± 0.09	0.54 ± 0.02	0.12 ± 0.02
glucose 1-phosphate kinase	5.32 ± 0.31	2.46 ± 0.19	5.71 ± 0.16	0.44 ± 0.05
glucose 1,6-diphosphate synthase	3.87 ± 0.16	3.36 ± 0.19	10.55 ± 0.26	2.26 ± 0.18
fructose 1,6-bisphosphate dependent glucose 1,6-bisphosphate synthase	1.46 ± 0.08	0.92 ± 0.05	0.04 ± 0.04	0.67 ± 0.05

With four enzymes synthesizing Glc-1,6P2 and it not being metabolized, there needs be a way to degrade it so that the cell maintains proper Glc-1,6P2 concentrations. The only observed reaction known to degrade Glc-1,6P2 is carried out by *glucose 1,6-bisphosphatase*, Equation 5.

Equation 5 – reaction catalyzed by *glucose 1,6-bisphosphatase (5)* (E.C. has not been assigned)

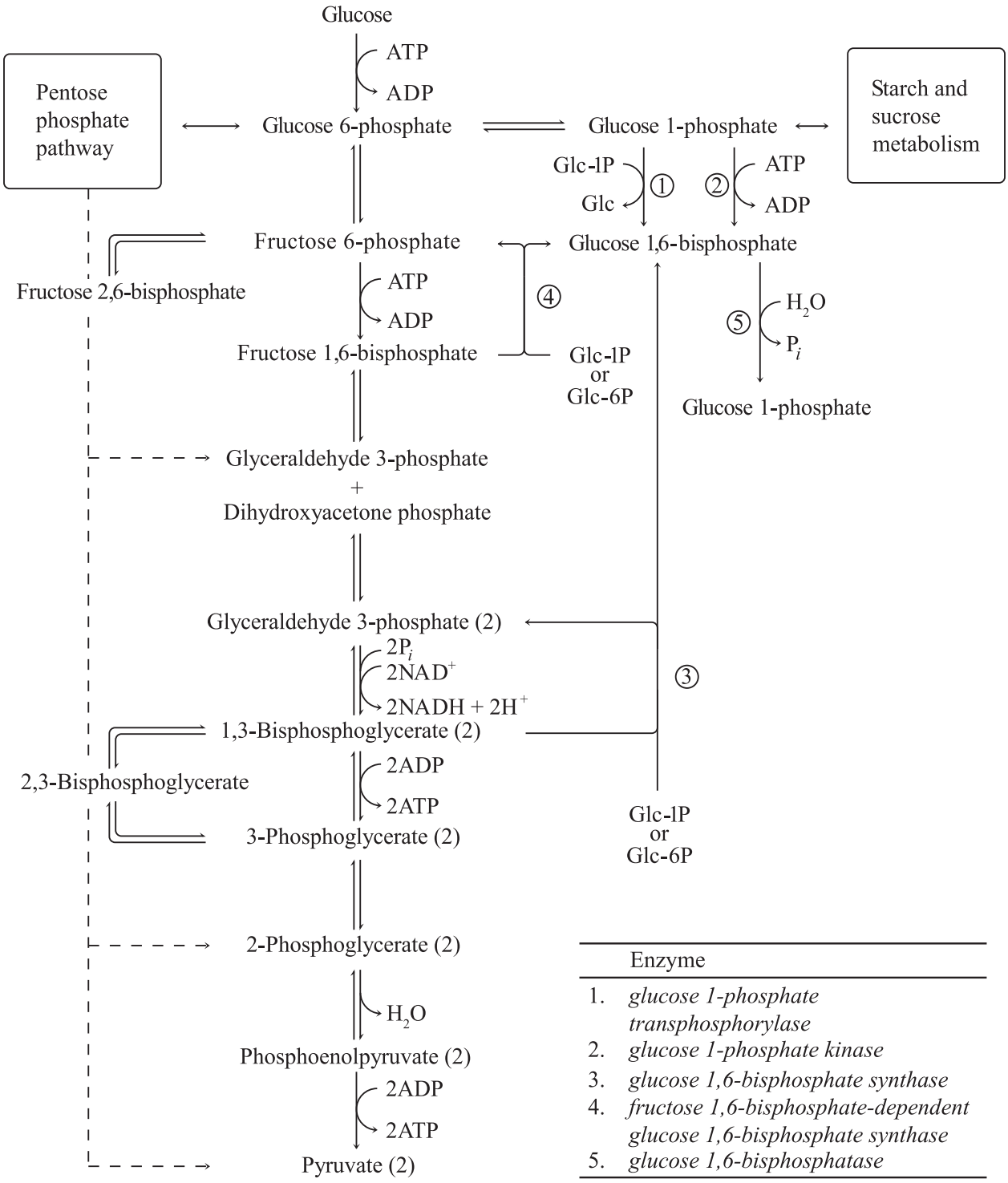
$$\text{Glc-1,6P2} + H_2O \rightarrow \text{Glc-1P} + P_i$$

Two phosphatase isoforms of this enzyme exist, one in the brain and one in muscle tissue. Because the brain *(24)* and muscle *(25)* phosphatase isoforms respond differently to Ca^{2+} and calmodulin, they were determined to be different isoforms and not the same enzyme. Van Schaftingen et al. *(26)* identified the brain isoform in 2008 to be *phosphomannomutase 1*, *PMM1*. Our work has been focused on characterizing and isolating the muscle phosphatase.

With the background information about the enzymes that synthesize and degrade Glc-1,6P2, it is now appropriate to look at the more complete picture of glycolysis, as illustrated in Figure 2.

Figure 2 illustrates that Glc-1,6P2 is intricately involved in glycolysis. This, taken with the regulatory role of Glc-1,6P2 implies that Glc-1,6P2 concentrations should be affected by changes in glycolytic flux. Several studies have been performed to observe the concomitant changes in Glc-1,6P2 concentrations as glycolytic flux is altered. These results clearly show the importance of Glc-1,6P2 in metabolism.

Figure 2. More complete picture of glycolysis, highlighting the importance of Glc-1,6P2.



	Enzyme
1.	*glucose 1-phosphate transphosphorylase*
2.	*glucose 1-phosphate kinase*
3.	*glucose 1,6-bisphosphate synthase*
4.	*fructose 1,6-bisphosphate-dependent glucose 1,6-bisphosphate synthase*
5.	*glucose 1,6-bisphosphatase*

Katz et al. *(27)* compared the change in concentrations of Glc-1,6P2 and various metabolites at rest, 20 seconds after muscle contraction, and at muscle fatigue. They observed an increased concentration of all the glycolytic metabolites tested and also an increased Glc-1,6P2 concentration both after 20 seconds of contraction and during muscle fatigue when compared to the concentrations of these compounds at rest.

Rose et al. *(28)* tested the concentration of Glc-1,6P2 in *pyruvate kinase* deficient red blood cells. In attempts to overcome the deficiency of *pyruvate kinase*, cells would be expected to have increased concentrations of the glycolytic intermediates prior to this enzyme in efforts to increase flux through the pathway. Rose et al. does not report the concentrations of glycolytic intermediates, but they do report a 2 to 3 fold increase in Glc-1,6P2 concentrations in these cells.

The concomitant changes in Glc-1,6P2 concentrations were also observed in a variety of other situations, including in response to selected hormones, various disease states, and in the presence of other compounds that regulate glycolytic flux. Glc-1,6P2 concentrations increased in response to insulin, epinephrine, glucose, and cyclic AMP. Conversely, Glc-1,6P2 concentrations were lower in diabetic and fasted states and when cyclic GMP levels were high. These studies are referenced in Table 4, partially adapted from Beitner *(29)*.

The previous examples represent situations in which concomitant changes in Glc-1,6P2 concentrations are reported as a result of changes caused by a variety of effectors. Piatti et al. *(43)* tested the converse approach. They altered cellular concentrations of Glc-1,6P2 in red blood cells and measured glucose consumption. In their study, Glc-1,6P2 concentrations were increased in red blood cells by two different methods. The first method involved encapsulating Glc-1,6P2 into cells and resulted in a six-fold intracellular increase in Glc-1,6P2 concentrations. The second method involved encapsulating the enzyme *glucose 1,6-bisphosphate synthase* into

cells and resulted in a two-fold intracellular increase in Glc-1,6P2 concentrations. They reported that in the six-fold and two-fold increased Glc-1,6P2 systems, glucose consumption decreased by approximately 50% and 25%, respectively.

Table 4. Concomitant changes in Glc-1,6P2 concentrations as a result of various effectors. Partially adapted from Beitner *(29)*.

Effectors	Glc-1,6P2 concentrations	Tissue type - reference
Compounds		
cyclic GMP	decrease	M *(25)*, D *(30)*
Ca^{2+}	decrease	M *(25)*
lithium	decrease	M *(31)*, B *(31)*, L *(31)*
anesthetics	decrease	D *(32)*
lysolecithin	decrease	D *(33)*
glucose	increase	P *(34)*
cyclic AMP	increase	M *(25)*, D *(35)*
Hormones		
vasopressin	decrease	M *(25)*
serotonin	decrease	M *(36)*, S *(36)*
bradykinin	decrease	M *(37)*
insulin	increase	M *(38)*, D *(38)*, F *(39)*
epinephrine	increase	M *(25)*, D *(35)*
Disease states		
anoxia	decrease	D *(6)*
diabetes	decrease	M *(38)*, D *(38)*
ischemia	decrease	B *(4)*
muscular dystrophy	decrease	M *(40)*
pyruvate kinase deficiency	increase	E *(28)*
Additional conditions		
old age	decrease	M *(41)*, S *(42)*
phospholipase A2	decrease	D *(33)*
fasting	decrease	M *(40)*
refeeding	increase	M *(40)*
muscle contraction	increase	M *(27)*

M: muscle; D: diaphragm; B: brain; S: skin; L: liver; P: pancreatic islets; E: erythrocytes; F: fibroblasts

These studies, and the additional studies referenced in Table 4, show that Glc-1,6P2 is deeply involved in glycolysis. However, our interest in understanding the role of Glc-1,6P2 stems from results obtained by Beitner *(44)* and Passoneau *(4)* wherein they indicate abnormal Glc-1,6P2 concentrations in dystrophic muscle and ischemic tissues, respectively. Our work was designed to determine whether restoring Glc-1,6P2 concentrations to their normal concentrations would reverse the disease state and act as a treatment.

Neither of the phosphatases nor any of the enzymes that synthesize Glc-1,6P2 was well characterized or identified at the inception of this project. Because of this, we had to determine how we wanted to focus our efforts. We chose to work on the characterization and identification of the muscle isoform of *glucose 1,6-bisphosphatase* for two main reasons. First, Beitner *(44)* reported that the muscle phosphatase isoform was over-active in dystrophic mice. They observed a greater than two-fold increase in enzymatic activity in muscular dystrophic mice aged four weeks and greater than three-fold increase in activity in mice aged eight weeks. We hypothesized that if the phosphatase enzyme was over-active, identifying an inhibitor for the enzyme might serve to restore Glc-1,6P2 concentrations to their proper levels. Second, it seemed more feasible to focus on the phosphatase because there were fewer known phosphatases than enzymes that synthesize Glc-1,6P2. If possible, we wanted to avoid having a second isoform compensate for the one we were studying.

This project was designed with hopes to benefit mankind rather than to just generate data and produce a degree.

Chapter 2

Preparing a Protocol

Detection Methods, Sample Preparation, Buffers, and Separation Techniques

Detection Methods

The first obstacle to overcome in developing a method to characterize and isolate *glucose 1,6-bisphosphatase* was to identify a reliable method to quantify enzymatic activity. We attempted to use various methods and instruments to directly quantify Glc-1,6P2, Glc-1P, and P_i. Additionally, we attempted to use Glc-1P to develop an indirect method to quantify enzymatic activity. Each of the unsuccessful detection methods will be described briefly. Eventually we were able to develop an assay to quantify phosphatase activity. This technique will be described in more detail.

$NADP^+$/NADPH

Multiple groups used the $NADP^+$/NADPH assay developed by Passoneau et al. *(4)* in efforts to identify *glucose 1,6-bisphosphatase*. Initially, we used this method as well. We also regressed back to using this assay when other detection methods seemed to fail.

The $NADP^+$/NADPH assay is a spectrophotometric method that detects NADPH concentrations by measuring the absorbance of a sample at 340 nm. 340 nm is the optimal detection wavelength because there is virtually no background from $NADP^+$ at this wavelength. This method is an indirect method that allows the observer to detect phosphatase activity by measuring the formation of NADPH. NADPH is formed when the phosphatase reaction is coupled with a sequence involving two other enzymatic reactions, see Equations 5 – 7.

Equation 5 – reaction catalyzed by *glucose 1,6-bisphosphatase*

$$\text{Glc-1,6P2} + H_2O \rightarrow \text{Glc-1P} + P_i$$

Equation 6 – reaction catalyzed by *phosphoglucomutase*

$$\text{Glc-1P} \rightarrow \text{Glc-6P}$$

Equation 7 – reaction catalyzed by *glucose 6-phosphate dehydrogenase*

$$\text{Glc-6P} + NADP^+ \rightarrow \text{6-phosphoglucono-}\delta\text{-lactone} + \text{NADPH}$$

Because this assay was difficult to use, did not give reproducible results, and did not allow for direct detection of either a reactant or one of the products, another detection method was sought.

Phosphorous-31 NMR

Phosphorous-31 NMR had some potential as a detection method for this project because it would theoretically allow us to detect both the reactant and products simultaneously. Preliminary results using this method were promising because Glc-1P, Glc-6P, and P_i were all distinguishable by chemical shifts. However, in relation to this project, this technique was not feasible. Glc-1,6P2 was too expensive to use at the concentrations required for detection under a modest time frame. As such, this technique was quickly abandoned.

However, this technique did serve a useful function. Since we were able to distinguish between Glc-1P, Glc-6P, and P_i we attempted to use this technique in a one-time experiment to confirm the published enzymatic reaction *(5)* performed by *glucose 1,6-bisphosphatase*. This was desirable because of a recent publication by Van Schaftingen et al. *(26)* wherein they indicated that it was impossible to determine whether the brain isoform of the phosphatase formed Glc-1P or Glc-6P because *PMM1* retains some *phosphoglucomutase* activity. The results from this study will be discussed in the Chapter 4.

Mass Spectrometry

Efforts were also invested into using mass spectrometry as a viable detection method for the enzymatic assay. Advantages to using this as a method of detection included low detection limits and the potential to identify both Glc-1,6P2 and Glc-1P. Much time was spent trying to identify a good mobile phase solvent to get these two chemical species to fly in the mass spectrometer consistently and yield repeatable results. Attempts were unsuccessful, likely

because both of these compounds are charged species under normal conditions, and mass spectrometry is more effective when the compound is not charged to begin with.

Refractive Index Detector

A refractive index detector was an attractive alternative because it is a general detector, responding to nearly all solutes. It is also reliable. One major drawback is that it is not as sensitive as other detection methods. When coupled with HPLC, this detector was not sensitive enough to detect Glc-1,6P2 and Glc-1P at the concentrations at which we were working. Consequently, this detection method did not meet our needs and was abandoned.

Evaporative Light Scattering Detector

An evaporative light scattering detector is also a general detector. It works by detecting the scattered radiation from laser light after it has passed through a nebulized cloud of analyte particles. A major benefit of this detector is that it detects nonvolatile compounds. This detector has been used to identify carbohydrate compounds in the past, so it had an apparent appeal.

After repairing the ELSD in the lab, it was quickly determined that we were operating below the limit of detection for the system. Coupling this detector with an HPLC diluted the sample too much to detect low concentrations of Glc-1,6P2.

High Performance Thin Layer Chromatography

HPTLC was another option as a detection method. Advantages included the potential to separate Glc-1P from Glc-1,6P2. A major disadvantage of this technique is that it is not quantitative. However, in efforts to identify *glucose 1,6-bisphosphatase*, only qualitative results were required. The formation of Glc-1P would have been supporting evidence of the phosphatase reaction. This method would have functioned in aiding to isolate the phosphatase.

Various staining techniques were performed in conjunction with the HPTLC plates to determine if Glc-1,6P2 and Glc-1P could be detected, in addition to identifying a mobile phase for separation. Unfortunately, a suitable staining solution and separation protocol could not be determined.

Ammonium Molybdate

We explored using another protocol involving ammonium molybdate published by Dunham and Christianson *(45)* as a spectrophotometric method to measure the amount of free phosphate produced as the reaction proceeded. Absorbance was measured at 820 nm using a spectrophotometer. This method showed some promise; however, it was determined that this method did not produce reproducible results.

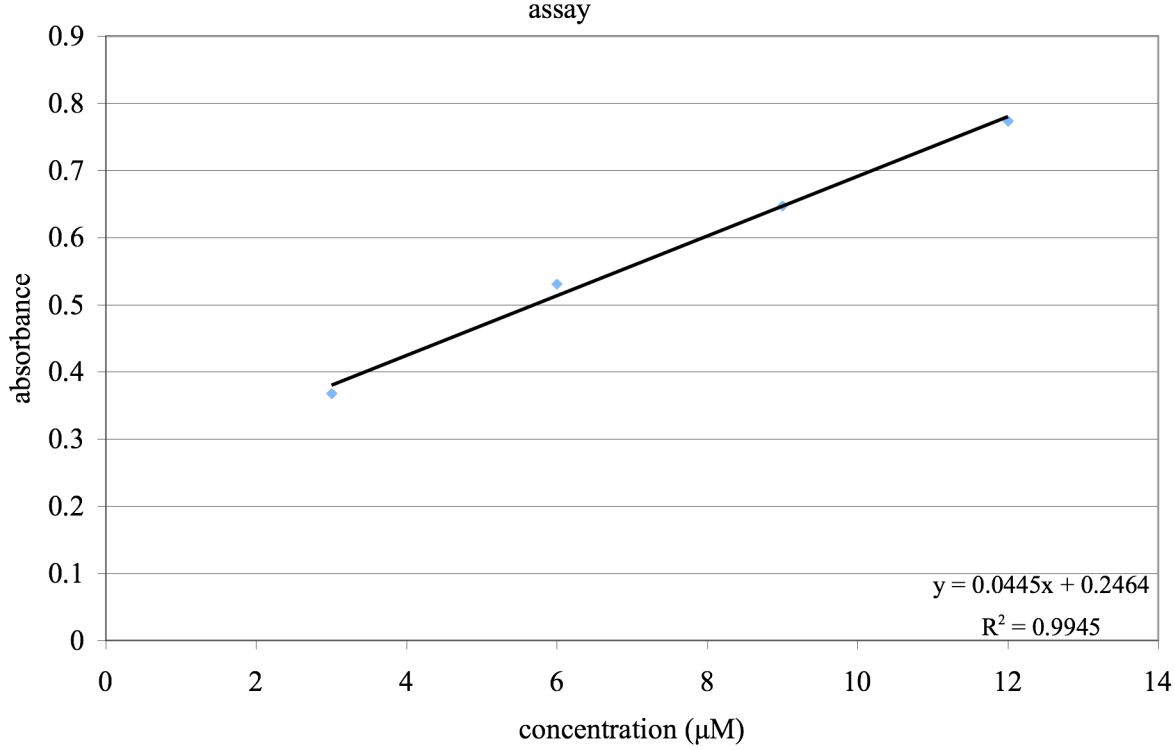
AM/MG (Ammonium Molybdate/Malachite Green)

The AM/MG assay used by Bassols et al. *(46)* quickly became the detection method of choice once it began to be used in this lab. The AM/MG assay works by spectrophotometric detection of the absorbance of light at 620 nm, the optimal detection wavelength for this assay. P_i was captured inside a keggin cage, formed by the molybdate, and quantified using malachite green. The greater the concentration of P_i absorbed, the greater the absorbance.

Preliminary experiments showed some difficulties using this assay because the malachite green was contaminated with P_i. Eventually we went to a commercial AM/MG solution (PiBlue Phosphate Assay Kit, BioAssay Systems) that allowed for phosphate analysis. This detection method showed that quantitative results could be achieved. Figure 3 shows that the AM/MG assay had a linear response to a P_i standard. However, this assay was not without its problems. It would occasionally form a precipitate, rendering the assay ineffective with certain protein samples. We were not able to identify what caused the precipitation. The only option was to

prepare a new protein sample. Unfortunately because of the crudeness of the sample prep, this was often required.

Figure 3. Calibration curve using a phosphate standard with the AM/MG assay



Sample Preparation

A major goal of this project was to identify the amino acid sequence of *glucose 1,6-bisphosphatase* so that it could be cloned in the future. Work towards the isolation of *glucose 1,6-bisphosphatase* was carried out with a few of the detection methods mentioned in the previous section. Because of false positives, work did not progress. The methods that gave false positives were the NADP+/NADPH assay and the AM/MG non-commercial assay. It was only later that it was determined that these detection methods were giving false positives, and that we needed to make substantial changes to advance the project. The results from these analyses will be discussed later. First, a discussion on the types of samples used and their advantages will follow.

A variety of sample sources were used in efforts to identify the best source tissue for purification and characterization. Source tissues tested were cow muscle, cow liver, chicken breasts, pork muscle, human erythrocytes, and *E. coli*. With exception of *E. coli* and the human erythrocytes, fat was removed from the samples before they were homogenized with a buffered solution, centrifuged, and filtered through glass wool to prepare them for separation. In all instances, the supernatant was retained as the sample. Dialysis to remove excess P_i typically followed ammonium sulfate precipitation, which will be discussed in the separation portion. *E. coli* cells were cracked with a French press and centrifuged to remove cell debris. They were then dialyzed against a buffer to remove excess P_i. Erythrocyte samples were prepared from human blood. The blood was collected in either heparin or EDTA to prevent clotting and centrifuged to collect the erythrocytes. The erythrocytes were then washed three times with PBS, lysed with ice water, and dialyzed against a buffered solution.

Cow Muscle

The sample of choice for this lab was cow muscle. Several sample preparations were performed with cow tissue. Advantages to using cow tissue included the ability to get fresh tissue from the Deseret Meat Co. slaughterhouse. It was free, and easy to work with. The best sample containing *glucose 1,6-bisphosphatase* was obtained from cow tissue. This sample will be discussed in further detail later.

Chicken Breasts

In efforts to reduce the initial amount of hemoglobin in the sample, we explored using chicken breasts as the source tissue instead of cow muscle. However, because it was not as easy to get fresh chicken samples and there was no other observed benefit to using chicken as the source tissue, it was not used as frequently as cow muscle.

E. coli

E. coli was an attractive sample source because it is a significantly less complex system than the other sample sources. We hypothesized that we may be able to use *E. coli* as a sample source because Glc-1,6P2 plays such a prominent role in glycolysis. We hoped too that the *E. coli* sample would be the same isoform found in muscle tissue. Unfortunately, we could not prevent the AM/MG assay from causing precipitation when we added it to *E. coli* samples. Consequently, *E. coli* was not a viable sample source.

Cow - liver

Ueda *(47)* used cow liver as a sample source. We attempted to use this too. However, it was extremely difficult to work with. Because of the composition of the liver, centrifugation after homogenizing did not produce a pellet of cellular debris to remove from the sample. Consequently, we could not prepare a sample ready for the next stage where we could attempt purification techniques.

Pork Muscle

We attempted to use a pork sample because they were being slaughtered at the slaughterhouse instead of cows one week. Pork was not suitable for our purpose, however. Sample preparation took considerably longer because of the large amount of fat on the sample and there was no observable advantage to using this as a source tissue over cow muscle. Taken with the fact that the slaughterhouse didn't kill pigs as often as cows, fresh pork samples were more difficult to obtain.

Human Erythrocytes

Erythrocytes are simpler than other tissues. Consequently, attempts to use erythrocytes as a sample source was attractive. However, a major disadvantage to using erythrocytes was that they contain a large amount of hemoglobin. Attempts to remove hemoglobin from *glucose 1,6-bisphosphatase* were unsuccessful. We attempted both size exclusion and $ZnSO_4$ precipitation. Hemoglobin eluted through a size exclusion column at approximately the same time as *glucose 1,6-bisphosphatase*. Attempts to remove hemoglobin via $ZnSO_4$ precipitation were also unsuccessful because so many other enzymes dropped out of solution that it rendered the sample ineffective.

Buffers

Attempts were made to find the optimal buffer to both isolate and characterize *glucose 1,6-bisphosphatase*. Following are some of the buffers used. As progress was made, it was determined that simpler buffers could be used than those previously identified in other papers. Where applicable, buffers used by other groups are cited. Buffer 5 eventually became the buffer of choice for sample preparation.

Buffer 1 – buffer used by Bassols et al. *(48)*
Meat homogenized in 10 mM triethanolamine, 1 mM EDTA, 0.125 M sucrose, 2 mM β-mercaptoethanol then added to the following buffered solution
0.5 mM KCl, 10 mM $MgCl_2$, 20 μM EGTA, 0.5 M hepes, 0.1 M DTT, pH's ranging from 7.0 to 7.5

Buffer 2 –
0.5 mM KCl, 10 mM $MgCl_2$, 20 μM EGTA, 0.5 M hepes, 0.1 M DTT, pH's ranging from 7.0 to 7.5

Buffer 3 –
0.1 M phthalic acid, pH's ranging from 5.5 to 6.5

Buffer 4 – buffered used by Van Shaftingen (identified *PMM1*) *(26)*
50 mM tris, 0.1 mM EGTA, 5 mM $MgCl_2$, pH 7.1

Buffer 5 – similar to buffer used by Beitner *(44)*
10 mM tris, pH's ranging from 7.0 to 7.5

Buffer 6 –
10 mM bis tris propane, pH's ranging from 7.5 to 9.45

Separation Techniques

Multiple separation techniques were used in attempts to purify *glucose 1,6-bisphosphatase*. Following is a brief description of the techniques, including some of the advantages they offer.

Ammonium sulfate precipitation

Ammonium sulfate precipitation is a protein purification technique that relies on salting out proteins by changing their solubility by the addition of a salt. Ammonium sulfate is the salt of choice for most biological applications because of its high solubility. This technique is considered a harsh technique because many proteins are unable to withstand the change in solubility and become partially or completely unfolded. This is particularly true for enzymes. The change in solubility often results in inactivated enzymes.

Ammonium sulfate precipitation is typically used as a first step to remove large quantities of undesired proteins from the protein of interest. As each protein is affected differently by changes to its ionic surroundings, they precipitate out at various concentrations of ammonium sulfate.

For simplicity in referring to samples that underwent ammonium sulfate precipitation, a P will just follow the percentage. For example, a 20% ammonium sulfate precipitation will just be read 20P. If there is a range, it will be read 70-80P. This would be for a sample that was brought to 70%, the precipitate was removed and the sample was brought up to 80%, thereby resulting in a precipitation that only contains the proteins that precipitate between 70% and 80%.

Spin filters – ultrafiltration

Spin filters are designed to allow only certain sized compounds through them. They are crude and inaccurate, in that they rarely actually separate at the specified molecular weight cut

off. However, they are still useful despite the inconsistencies and only allow compounds that are smaller through them. When various filter sizes are used in tandem with each other, they can give an approximate molecular weight for a compound. Various filters (30, 50, and 100 kDa) were used to help predict the molecular weight of *glucose 1,6-bisphosphatase* and aid in purification.

Tube gel – size exclusion apparatus

The tube gel apparatus used in our lab was a Bio-Rad Model 491 Prep Cell. A tube gel is designed to separate protein samples by continuous-elution electrophoresis. The procedure for running a tube gel is similar to running other gels in that the sample is run through a gel matrix for separation. However, unlike standard gel electrophoresis, the matrix is designed to prevent proteins from denaturing, and also allows for large-scale separation. The tube gel is thermally controlled to produce a uniform separation throughout. Proteins are separated based on their size and charge.

Gel electrophoresis

In gel electrophoresis, after precautions have been taken to remove interactions between the sample and stationary phase often by adding SDS, an electrical field is applied to a gel containing a protein sample. The proteins in the sample are separated by their molecular weight. The gel can then be stained to show how far the sample migrated and the relative purity of a sample. Most electrophoresis apparatuses allow for multi-well analysis. Typically a standard containing known proteins is run in an adjacent well to indicate the approximate molecular weight of the sample.

Rotofor cell – isoelectric focusing

A rotofor cell takes advantage of the isoelectric point (pI) of an enzyme in order to separate it from other proteins. When a rotofor cell is operated, an electric field is passed through a multi-buffered solution. The buffers create a pH gradient. Individual proteins then migrate to the pH that corresponds to their pI. In this way proteins can be separated from one another according to their pI. The rotofor cell apparatus in our lab was a Biorad rotofor cell. It created a buffered region ranging from pI values of 3.9-9.5 that could be collected into 20 fraction compartments.

Size exclusion – HPLC and FPLC

Size exclusion columns separate samples based on size. We attempted to use two varieties of size exclusion columns to isolate *glucose 1,6-bisphosphatase*. One was an analytical column and the other was a preparatory column that separated large samples.

The analytical column was a 25 cm Macrosphere GPC 300 angstrom analytical size exclusion column that was attached to an HPLC instrument.

The preparatory column was a Superdex 200 10/300 GL GE Healthcare column, which was coupled to an Aktapurifier 100 FPLC.

DEAE – anion exchange chromatography

DEAE is a type of anion exchange chromatography, which separates proteins based on their charge. DEAE columns have a positively charged resin (amine) that retains negatively charged proteins. At a given pH, negatively charged proteins are retained on the column, while neutral or positively charged species flow through. The retained species are later removed from the column by inducing a salt gradient, which allows them to come off the column.

DEAE serves as a good resin for ion exchange chromatography because it has a pKa of 11.5. This allows it to withstand a large pH range, providing the user the ability to change the pH

of the protein sample to determine what optimal pH value to use for their separation. We tested various pH's, ranging from 7 – 9.45. Initial results indicated that the phosphatase was not retained on the column at a pH below 7, so lower pH's were not analyzed.

CM – cation exchange chromatography

CM is another type of ion exchange media produced by Bio-Rad. However, its resin is negatively charged (carboxylic acid), and thereby retains positively charged proteins. This particular resin is used to make weak cation exchange columns. Our CM column was purchased from Bio-Rad and was the Econo-Pac CM 1 x 5 mL size.

High S – cation exchange chromatography

High S media is similar to CM media in that it is also used as the resin for a cation exchange column. However, it is considered to be a strong cation exchange media (sulfite). The High S column we used was purchased from Bio-Rad and was the Econo-Pac High S 1 x 5 mL size.

Hydrophobicity columns

Hydrophobicity columns separate samples based on the amount of hydrophobic interactions between the resin and the surface of the biomolecule. They separate similarly to a reverse-phase column, but use milder elution conditions making denaturization less of an issue. However, it is difficult to predict whether a specific hydrophobicity column will retain a protein. Consequently we tested six hydrophobicity columns to determine if they would retain *glucose 1,6-bisphosphatase*. These columns were purchased from Sigma and were the phenyl FF (low sub), butyl FF, octyl FF, phenyl HP, butyl-S FF, phenyl FF (high sub).

$ZnSO_4$

Low concentrations of $ZnSO_4$ can be used to precipitate certain proteins out of solution. $ZnSO_4$ was used to remove hemoglobin from protein samples.

Reverse phase chromatography (C18)

Reverse phase column chromatography is a well-developed analytical tool. Larger preparatory columns are used to purify protein samples. Hydrophobic interactions from the stationary phase interact with the proteins to retain them on the column, allowing for separation. A major advantage of C18 columns is their universality.

Glucosamine affinity column

Affinity columns are a highly specific separation method. The columns are derivatized, and reversibly bind proteins that have a strong interaction with the resin. The proteins are eluted off when the binding chemical is added to solution. This separation technique is advantageous because it allows for large-scale, highly specific purification and it concentrates samples.

Preliminary results indicated that glucosamine partially inhibited *glucose 1,6-bisphosphatase*. Therefore, we made a glucosamine affinity column because we thought it might bind to the phosphatase and thereby retain the enzyme, greatly purifying our sample.

Chapter 3

Pitfalls and Difficulties of Isolating and Characterizing *Glucose 1,6-bisphosphatase*

Much of the research for this project built on itself. One experiment would direct to another avenue or another test. While thousands of experiments were performed to view enzymatic activity or to prepare another tissue sample, not all of these experiments will be included in this section. This section is designed to illustrate the work that was done in attempts to identify methods to prepare protein samples, detect enzymatic activity, and isolate *glucose 1,6-bisphosphatase*. Not all the work illustrated in this section is chronological, but is organized to give a better understanding of what was accomplished.

At the time of the experiments, the results from these experiments may have seemed promising, but later experiments provided contradictory results. This section contains the results from multiple separation techniques and detection methods; however, the most reliable results will be discussed in the next section.

$NADP^+$/NADPH – cow muscle and liver

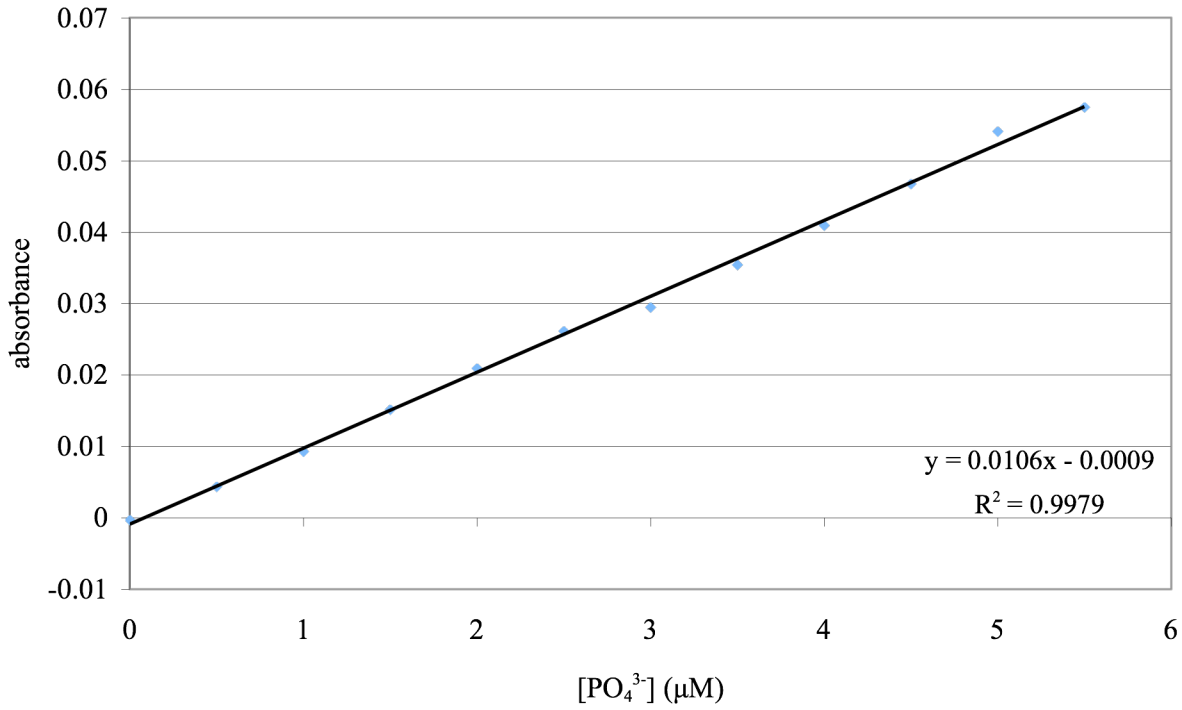
Several groups used $NADP^+$/NADPH to detect phosphatase activity indirectly. This was the detection method being used when I began my work in the Herron lab. Inconsistencies with the assay and multiple cow muscle and liver samples made us realize that this assay had some downsides in addition to being an indirect method to detect phosphatase activity. This detection method was therefore abandoned for a while. These difficulties led us to explore other methods of detection.

Non-commercial AM/MG assay – calibration curve and precipitation problem

A calibration curve for the non-commercial AM/MG solution was prepared to determine if this assay was a reliable detection method to quantify the amount of P_i in solution. Figure 4 illustrates that curve. These results indicated that a linear response to P_i using the AM/MG assay could be achieved. We tried to apply this assay to a protein sample to determine if this could be

used to help identify *glucose 1,6-bisphosphatase*. Cow muscle was prepared in buffer 1 and it was observed that a green fluffy precipitate formed. This was a major cause of concern and efforts to overcome this precipitation ensued. Longer centrifugation and the addition of SDS were two methods attempted to prevent the precipitation.

Figure 4. Calibration curve using a phosphate standard with the AM/MG non-commercial assay



Certain protein samples precipitated less than others, but we were unable to identify the cause of the precipitation. Overloading the assay with P_i did not cause a precipitate to form. This led us to believe that the sample itself was for some reason precipitating out. Because we could not identify the source of the precipitation, we eventually proceeded with our experiments. It was later determined that this detection method needed to be abandoned.

Non-commercial AM/MG assay – boiled supernatant as activator

Guha and Rose *(24)* reported that boiled supernatant removed from the ammonium sulfate precipitations contained an activator for the phosphatase. We attempted this procedure,

but did not observe any noticeable effect caused by the boiled extract. Consequently we did not proceed to use this method to help identify the enzyme.

Non-commercial AM/MG assay – ammonium sulfate precipitation test

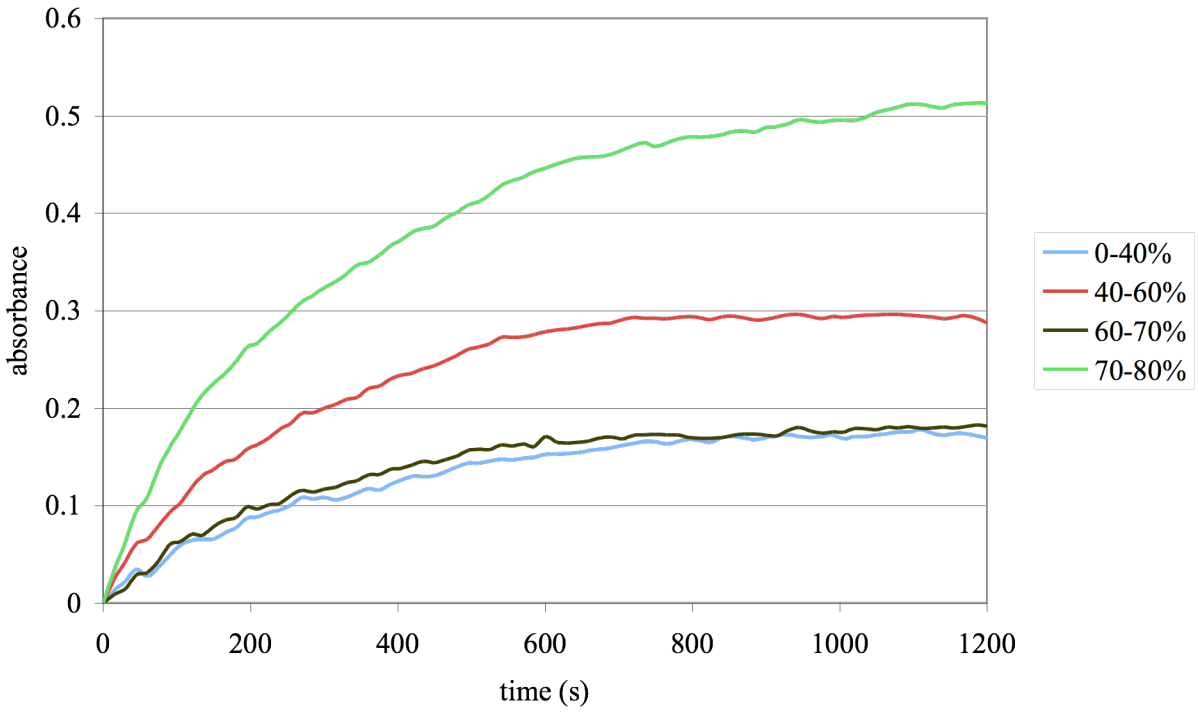
Bassols et al. *(46)* reported that *glucose 1,6-bisphosphatase* precipitated out with an 80P ammonium sulfate cut. After having prepared various tissue samples at this concentration of ammonium sulfate, we attempted to refine the ammonium sulfate protocol to identify a more compact range to precipitate out the phosphatase.

Cow muscle was homogenized and cell debris was removed by centrifugation. The sample was then precipitated with a 40P ammonium sulfate cut. The precipitate was resuspended and dialyzed against buffer, while the supernatant was brought up to a 60P ammonium sulfate concentration. This process was repeated with 70P and 80P ammonium sulfate cuts. In all, four cuts were made: 0-40P, 40-60P, 60-70P, and 70-80P.

These four samples were tested for phosphatase activity using the non-commercial AM/MG assay. The absorbance was measured over a range of 20 minutes, and the change of absorbance was indicative of phosphatase activity. Results from this experiment, Figure 5, indicated that the most active phosphatase activity was found in the 70-80P precipitation, with slight activity in the 40-60P fraction.

These results led us to hypothesize that there were potentially two isoforms of *glucose 1,6-bisphosphatase*.

Figure 5. Phosphatase activity following ammonium sulfate precipiation



Non-commercial AM/MG assay - filters

As there is no published work that reports the molecular weight of *glucose 1,6-bisphosphatase*, we attempted to use filters to help determine the approximate molecular weight and to partially purify our samples. A portion of the 70-80P protein sample was placed in a 100 kDa filter and centrifuged. The filtrate was placed on a 50 kDa filter and centrifuged. After doing appropriate washes to remove any compounds that passed through the filter, the samples were analyzed for activity. Despite problems with precipitation and incongruencies with the assay, the test indicated that both the sample with a MW greater than 100 kDa and the 50-100 kDa sample contained the phosphatase, while the sample below 50 kDa did not.

Non-commercial AM/MG assay – DEAE

A protein sample in buffer 1 was separated using a DEAE column. The DEAE separated the protein sample into four fractions. The two most retained peaks showed phosphatase activity.

These results indicate that *glucose 1,6-bisphosphatase* has a pI above 7.0, because it was retained on the column. However, there was precipitation in the vials, complicating the results obtained.

Mass spectrometry – tryptic digest

While the sample was still crude, samples separated on DEAE had shown significant purification. In response to these results, we digested our sample with trypsin and ran it on a mass spectrometer, ESI MS-MS, to try to identify the phosphatase. Table 5 is a list of the enzymes that were in solution (duplicates only included once), included in this table is the Mascot score, the higher the score the better the match. Certain enzymes stood out as having a higher probability of being a phosphatase. We specifically took note of any hypothetical proteins, other phosphatases, or phosphoglucomutases.

Table 5. Results from tryptic digest of sample purified by DEAE.

Enzyme	Mascot score
creatine kinase, muscle [Bos taurus]	676
serum albumin precursor (Allergen Bos d 6) (BSA)	643
cytoplasmic creatine kinases [Physeter catodon]	307
hypothetical protein LOC515067 [Bos taurus]	233
M-CK [Gallus gallus]	155
hemoglobin subunit beta-A (Hemoglobin beta-A chain) (Beta-A-globin)	132
hypothetical protein LOC513212 [Bos taurus]	78
alpha-fetoprotein	70
neuroleukin [Homo sapiens]	58
phosphoglucose isomerase-1 [Anguilla anguilla]	58

Non-commercial AM/MG assay – rotofor cell

The DEAE fraction with the greatest phosphatase activity was submitted to the rotofor cell to further purify the sample by pI. Unfortunately, one of the buffers used in the commercial solution contained P_i, causing such significant precipitation that the results were completely

invalid. Dialysis of the fractions was unsuccessful because of the small sample volume for each rotofor fraction. Using spin filters to remove the excess phosphate was also unsuccessful.

Non-commercial AM/MG assay – tube gel

The remaining DEAE sample that was not placed on the rotofor cell was placed on the tube gel, to purify the sample by size. The sample was too dilute after the tube gel to observe any phosphatase activity.

Non-commercial AM/MG assay – glucosamine tests

Because of the structural similarities shared by Glc-1,6P2 and glucosamine, we theorized that glucosamine might also bind in the binding pocket of *glucose 1,6-bisphosphatase*. Analysis indicated that glucosamine might act as an activator and as a competitive inhibitor, depending on the concentration of analyte. In response to these results, we made a glucosamine affinity column.

Non-commercial AM/MG assay – glucosamine affinity column

Not a single peak was detected as being retained by the glucosamine affinity column. Despite these negative results, all the fractions were tested using the non-commercial AM/MG assay. No phosphatase activity was observed. We concluded that we must have obtained false positives from the glucosamine tests, or that it cannot get into the binding pocket to interact with the phosphatase. These results led me to wonder as to the reliability of the assay as a detection method.

Ammonium molybdate – glucosamine affinity column

We analyzed the fractions from the glucosamine affinity column with the ammonium molybdate assay. Phosphatase activity from these fractions was not observed with this assay either.

Ammonium molybdate – zinc sulfate precipitation

Zinc sulfate at a concentration of 0.5 mM precipitates out hemoglobin. It was determined that slowly adding zinc sulfate resulted in precipitating out the red color. However, if the zinc sulfate were added too quickly, additional proteins would precipitate out with the hemoglobin. This technique was applied to a 60P ammonium sulfate sample to remove the hemoglobin. Analysis showed that the largest phosphatase activity precipitated out in this ammonium sulfate range, contrasting the results obtained from the non-commercial AM/MG assay indicating the 70-80P ammonium sulfate precipitation was the most active fraction.

Mass spectrometry

Because we wanted to confirm phosphatase activity more completely, we needed to identify a detection method that would allow us to quantify the amount of both product and reactant. We turned to mass spec. Efforts were placed into getting Glc-1P, Glc-6P, and Frc-1,6P2 to successfully fly in mass spec, as Glc-1,6P2 is expensive. We analyzed samples in both positive and negative modes to identify these compounds. Various solvent combinations were used in attempts to identify a working protocol. Our efforts were unsuccessful.

Ammonium molybdate – tube gel with stacking column

A tube gel with stacking column was prepared and an active 70P ammonium sulfate sample with a dye to stain proteins was loaded onto the column. The stacking column was used to keep the sample as concentrated as possible before separating it with the tube gel. This was desirable because a previous run indicated that the tube gel diluted the sample too much to observe phosphatase activity.

Even with the stacking column, the fractions were too dilute to detect any phosphatase activity. Efforts to concentrate the sample by filtering were also unsuccessful, as no phosphatase activity was observed.

Later analysis indicated some color change in some of the fractions two days after the samples were analyzed for phosphatase activity. These samples were analyzed by gel electrophoresis and mass spec after a tryptic digest. Because the samples were too dilute these two detection methods were unsuccessful and further separation using this sample was not possible.

Ammonium molybdate – E. coli

Because *E. coli* is a simpler system than cow tissue, we explored using it to isolate *glucose 1,6-bisphosphatase*. The *E. coli* were cracked in a French press and centrifuged to remove cell debris. Following this, the sample was placed on the DEAE column to further purify the sample. Each *E. coli* sample precipitated when the sample was combined with the ammonium molybdate solution. Despite the false positive caused by the precipitation, a tryptic digest was prepared to search for the identity. However, it was determined that these results were invalid because of the problems caused by the precipitation.

$NADP^+$/NADPH – E. coli

False positives caused by precipitation of the assay and samples led us to explore additional detection methods. We reverted back to the $NADP^+$/NADPH assay developed by Passoneau et al. and used earlier in this lab. This technique did not show phosphatase activity either.

Phosphorous NMR – E. coli

Difficulties with other detection methods led us to try to follow the phosphatase reaction using phosphorous NMR. We quickly determined that the concentration of Glc-1,6P2 was too low to yield results in a reasonable time period.

HPLC refractive index

We prepared various Glc-1P and Glc-6P solutions to determine whether we could detect these compounds using a refractive index detector. We also wanted to be able to separate these two species, if possible, to be able to confirm the published phosphatase reaction. It was quickly determined that the working concentrations of Glc-1P and Glc-6P were too dilute following HPLC to detect them at the assay conditions we were working under.

Non-commercial AM/MG assay – chicken

We returned to using the non-commercial AM/MG assay with a chicken sample. We hoped that changing the source tissue would allow us to circumvent the precipitation problem that we had originally seen with the non-commercial AM/MG assay. Ammonium sulfate precipitation, DEAE, and the glucosamine affinity columns were all tested as viable separation techniques for the chicken sample. Complications with precipitation still occurred even after the separation techniques were applied.

Evaporative light scattering detector

An evaporative light scattering detector was coupled with an HPLC equipped with a C18 column. Glc-1P, Glc-6P, Frc-1,6P2 samples were prepared and tested to determine if this technique would work as a suitable detection method. The peaks produced from these separations were broad, but Glc-1P and Glc-6P were observed to have different retention times. However, Glc-6P and Frc-1,6P2 eluted at the same time. The broad peaks and inability to separate Frc-1,6P2 from Glc-6P were deterrents, but we were still hopeful to create a better

method to resolve these issues. Unfortunately software incompatibility issues prevented us from attaining reliable/quantitative results. Using the front display to record data was also unreliable, not to mention tedious. This detection method had to be abandoned because of these difficulties.

Non-commercial AM/MG assay – heat resistance test

Results from Bassols et al. *(46)* indicating that *glucose 1,6-bisphosphatase* was relatively stable thermally led us to analyze whether we could use heat resistance as a purification technique. Samples were tested for phosphatase activity after being heated in a water bath at 60, 80, and 100 °C for 10, 15, and 10 minutes respectively. The results from our experiments were inconsistent with those reported by Bassols. We did not observe retained activity, and precipitation caused complications.

$NADP^+$/NADPH

Published results by Van Shaftingen et al. *(26)* indicating that they had isolated the brain isoform of *glucose 1,6-bisphosphatase* led us to change protocols in attempts to aid in the isolation of the muscle isoform. They monitored phosphatase activity using the $NADP^+$/NADPH assay and buffer 4.

After appropriate testing confirming that the assay appeared to be working, testing with protein samples began. Additional testing further confirmed complications with using the $NADP^+$/NADPH assay. There appeared to be a slight interaction between phosphoglucomutase and Glc-1,6P2.

Mass spectrometry and phenylhydrazine derivative test

The complications with the $NADP^+$/NADPH assay left us exploring other options as a means of detection. Using mass spectrometry as a viable detection method was again explored; we attempted to get Glc-1,6P2, Glc-1P, and Glc-6P to fly.

We also explored the possibility of derivatizing the carbohydrates to analyze them via mass spectrometry or colormetrically. Phenylhydrazine is a common derivatization technique for carbohydrates. After following a short procedure, carbohydrates turn a yellow color and form a precipitate. We performed phenylhydrazine derivative tests on Glc, Glc-1P, Glc-6P, Frc, and Frc-1,6P2. The phenylhydrazine derivative test did not work as well on the phosphorylated sugars as well as it did on the normal carbohydrates. The original procedure called for a large concentration of the analyte being tested. When efforts were made to miniaturize the procedure, no visible precipitation was observed. This technique was abandoned.

High performance thin layer chromatography

10 x 10 cm cellulose HPTLC plates were purchased from Merck, and their utility was examined to determine whether they could be used as a detection method. These plates were to serve as an alternative to bypass the precipitation effect that was occurring with AM/MG assay.

We experimented with various staining solutions to identify one that would stain P_i, Glc-1P, Glc-6P, and Glc-1,6P2. The ammonium molybdate, non-commercial AM/MG solution, and two general stains (p-anisaldehyde and cerium molybdate) were analyzed. We also experimented to identify a stain by making them with ammonium molybdate and various dyes (malachite green and brilliant blue). Each of these staining techniques was evaluated with and without the presence of heat, and with and without the presence of a black light.

After some apparent results, it was determined that the HPTLC did not suit our needs. This was around the time when the commercial AM/MG solution came in, which became the detection method of choice.

Summary of Results

Table 6 contains a summary of the results obtained from the experiments described in this section.

Table 6. Summary of Experiments and Results from Chapter 3.

Experiment title
$NADP^+$/NADPH – cow muscle and liver
Complications with assay – sought other detection method
Non-commercial AM/MG assay – calibration curve and precipitation problem
Calibration curve appeared linear – observed precipitation with assay
Non-commercial AM/MG assay – boiled supernatant as activator
Supernatant did not show activating properties as reported
Non-commercial AM/MG assay – ammonium sulfate precipitation test
Potentially two isoforms – 40-60P and 70-80P fractions – Figure 5
Non-commercial AM/MG assay - filters
Apparent mass of enzyme is above 50 kDa
Non-commercial AM/MG assay – DEAE
pI of enzyme above 7.0, but precipitation makes results inconclusive
Mass spectrometry – tryptic digest
Potential list of matches – Table 5
Non-commercial AM/MG assay – rotofor cell
Buffer contained phosphates, results invalid
Non-commercial AM/MG assay – tube gel
Sample too dilute to observe activity
Non-commercial AM/MG assay – glucosamine tests
Apparent interaction by glucosamine and phosphatase
Non-commercial AM/MG assay – glucosamine affinity column
Nothing retained on the column
Ammonium molybdate – glucosamine affinity column
Nothing retained on the column
Ammonium molybdate – zinc sulfate precipitation
Hemoglobin precipitated out
Mass spectrometry
Unable to identify a method to quantify Glc-1P, Glc-6,P, and Glc-1,6P2
Ammonium molybdate – tube gel with stacking column
Sample too dilute to observe activity
Ammonium molybdate – E. coli
Precipitation prevented analysis
$NADP^+$/NADPH – E. coli
No observed phosphatase activity

Phosphorous NMR – E. coli

Concentration of phosphate-containing compounds was too dilute for detection

HPLC refractive index

Sample too dilute to observe activity

Non-commercial AM/MG assay – chicken

Observed precipitation with chicken samples, similar to beef

Evaporative light scattering detector

Software incompatibility issues prevented analysis

Non-commercial AM/MG assay – heat resistance test

Did not appear to be thermally stable, precipitation problems

$NADP^+$/NADPH

Readdressed using this assay because brain isoform was identified, but we still saw complications

Mass spectrometry and phenylhydrazine derivative test

Derivative testing was unsuccessful as a detection method

High performance thin layer chromatography

Could not identify a suitable staining solution

Chapter 4

Promising Progress toward the Isolation and Characterization of *Glucose 1,6-bisphosphatase*

This section is describes the results that showed more potential than those in the previous section. While not all of the experiments discussed in this section worked, it provides information regarding the best sample that was prepared, the detection method of choice, and results from multiple separations techniques.

Old 40P sample

While working with the $NADP^+$/NADPH assay, we prepared a tissue sample that was eventually referred to in the lab as the old 40P sample. This sample was prepared using buffer 4 at a pH of 7.08. Special mention of this sample is being made because it was the best sample that we were able to prepare. Efforts to reproduce this sample were unsuccessful, and will be explained later.

This sample was prepared at a time when we were testing whether the order of performing certain experiments was important. Consequently, some of this sample was directly loaded onto the DEAE, some was loaded onto the rotofor cell, and some was precipitated with ammonium sulfate to a final concentration of 40P. All these samples were tested using the NADP+/NADPH assay. However, this assay was still unreliable, and was not giving decent results. The old 40P sample was therefore left in the fridge. It was tested months late and still showed phosphatase activity. The stability of this sample was uncommon. No visible precipitation was observed with this sample and the detecting solutions.

Commercial AM/MG assay

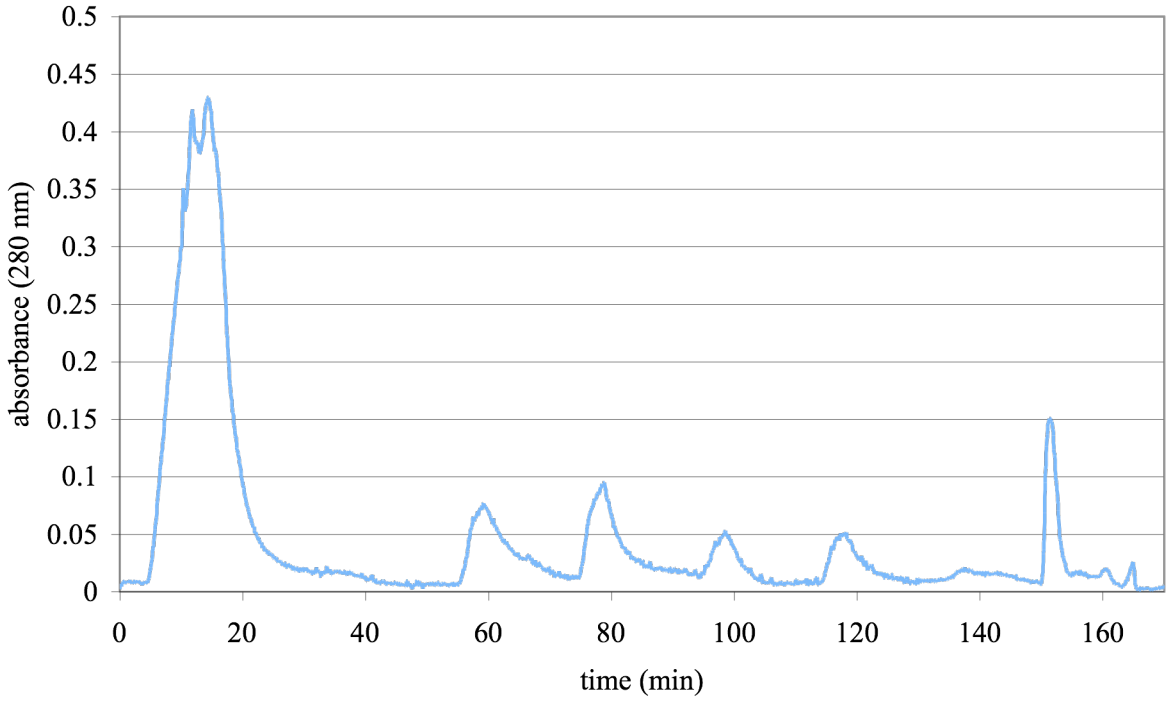
The first experiments ran with the commercial AM/MG assay (from here this will be referred to only as the AM/MG assay) were done to determine whether a calibration curve could be created with only P_i, and then whether it worked with a protein solution. The protein solution initially tested with the AM/MG assay was the old 40P solution as well as a 40-80P solution

made from the same sample at the same time the old 40P solution was made. The old 40P solution still showed active *glucose 1,6-bisphosphatase* activity.

AM/MG assay – DEAE – old 40P

Some of the old 40P sample was separated with a DEAE column. A chromatogram of this run is illustrated in Figure 6. Analysis using the AM/MG assay revealed that the column did not retain *glucose 1,6-bisphosphatase*.

Figure 6. Chromatogram showing the separation of the old 40P sample following separation on the DEAE ion exchange column



Later tests indicated that the solution lost phosphatase activity within a couple weeks of the DEAE separation. This observation sparked a memory of one line written in a paper by Bassols et al. *(46)*. In it they observed that *glucose 1,6-bisphosphatase* quickly lost function after being separated by DEAE. Analysis of the old 40P sample not placed on the DEAE indicated that the sample was in fact still active. The combination of the observation made by Bassols and

our results intimated two options, either work quickly after using the DEAE as a separation technique or stop placing samples on the DEAE.

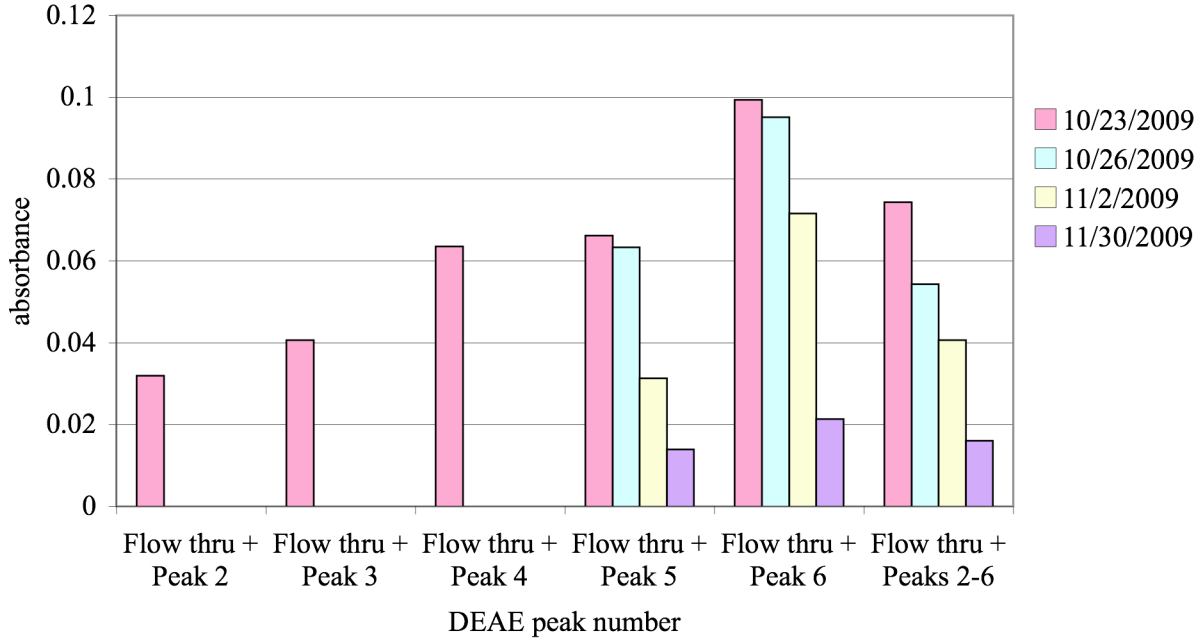
AM/MG assay – DEAE – old 40P sample – stabilization analysis

Since *glucose 1,6-bisphosphatase* became inactive shortly after being separated on the DEAE, we theorized that there might be a stabilization protein eluting off in one of the other DEAE fractions. After determining that recombining the DEAE fractions after the enzyme had become inactive did not restore activity, an experiment was designed to test the stability of the enzyme if the fractions were recombined immediately after the separation.

Five peaks were retained on the column, in addition to the un-retained peak that contained the phosphatase, as seen in Figure 6. The peak containing the phosphatase was recombined with each of the other peaks, and also with some of all the peaks. Each of these new samples was then analyzed for phosphatase activity over the course of the next month. The results from this test are shown in Figure 7. Since some of the peaks had already begun to show a sharp decline in activity, they were not analyzed for the entire month.

After the month was ended, phosphatase activity had decreased significantly in all the samples. However, recombining the phosphatase peak with another one of the fractions significantly reduced the deactivation of the phosphatase. We formed two theories to explain this observation. First, that because of dilution caused by the DEAE there was not a significant amount of the stabilization protein to prevent the phosphatase from denaturing. Second, that the separation reduced the effectiveness of the stabilization protein by causing it to slowly denature, resulting in a slow denaturization of the phosphatase.

Figure 7. Protein separated on DEAE column.
Recombined the flow thru (contains the phosphatase) with various DEAE fractions shocked off with salt (to identify stabilization protein).
Tested for phosphatase activity at the date specified.



Tryptic digest - DEAE – old 40P sample – stabilization analysis

The stabilization protein fractions then underwent tryptic digest and were analyzed by mass spectrometry to try to determine the sequence of the phosphatase. The results of this analysis were inconclusive. The mass spectrometer only detected the presence of trypsin.

AM/MG assay – ammonium sulfate precipitation test

The old 40P sample showed marked phosphatase activity, which was in contradiction to results obtained with the non-commercial AM/MG assay wherein the 40-60P and 70-80P fractions showed activity. The new assay system appeared to be more reliable, but tests still needed to be done to confirm the results. Therefore another sample was prepared, and precipitated out with ammonium sulfate. 0-40P and 40-80P fractions were made. After dialysis, analysis showed that neither of these two samples showed phosphatase activity. This was the beginning of trying to replicate the old 40P sample, but for some reason, it could not be

duplicated. Phosphatase activity was observed in other samples, but the stability of the solution could not be mimicked. The old 40P sample was stable for over a year, but other samples would not last even a month, if they even contained the phosphatase. We theorized that the old 40P might have a mutation that allowed it to be more stable.

AM/MG assay – analytical size exclusion column

A 25 cm Macrosphere GPC 300 angstrom analytical size exclusion column was attached to an HPLC instrument and old 40P sample was loaded onto the column. Fractions were obtained by hand every 5 drops for the first 45 fractions and four ≈1.5 mL fractions were captured at the end of the run to ensure that the sample was collected. The UV detector on the HPLC was not sensitive enough to detect any proteins eluting from the column, but we still decided to analyze each fraction with the AM/MG assay. This analysis did not reveal phosphatase activity. The sample did not appear to be concentrated enough to purify the enzyme with this column.

AM/MG assay – C18 protein preparative column

Old 40P sample was loaded onto a C18 protein preparative column coupled to an HPLC. The UV detector barely registered changes in absorbance while monitoring for proteins. Each fraction obtained was analyzed for phosphatase activity, but no phosphatase activity was observed. The sample appeared to have been diluted too much by the separation technique.

AM/MG assay – DEAE – C18

Another 40P ammonium sulfate sample was prepared, dialyzed, and separated using DEAE. The sample was filtered to concentrate the sample and loaded onto the C18 protein preparative column. This was done quickly so enzymatic denaturization would not be observed because of separating the sample with the DEAE column. Despite attempting to concentrate the

sample about 15 fold, the sample was still too dilute to detect the protein separation, and the AM/MG analysis did not yield results.

AM/MG assay – activator and inhibitor tests

Old 40P sample was used to try to identify activators and inhibitors. Sampling was done in a 96 well plate. A major objective of the project was to identify activators and inhibitors of *glucose 1,6-bisphosphatase*. Over 100 compounds were tested. Because of limited quantities of old 40P, additional compounds could not be detected. Other sample preparations were not as clean as the old 40P sample and often precipitated with the AM/MG assay. Because of this, efforts to identify activators and inhibitors were abandoned, until a more reliable protocol could be obtained to get good samples.

A list of the compounds analyzed to determine whether they affected the activity of *glucose 1,6-bisphosphatase* can be found in Supplemental Table A. The corresponding results are plotted in Supplemental Figure A. None of these compounds showed significant signs of being activators or inhibitors. It was determined that a few may act as potential regulatory compounds, but additional tests in the spectrophotometer revealed that the differences in the 96 well plate were fluctuations in the noise region which made it impossible to definitively discern that they regulated the phosphatase. Other experiments indicated that Frc-1,6P2 might to act as an inhibitor to *glucose 1,6-bisphosphatse*.

AM/MG assay - DEAE – old 40P sample – stabilization analysis with dialysis

We attempted to determine whether a stabilization protein was slowly denatured because the sample was in a saline solution when it came off the DEAE. We followed the same DEAE separation protocol as before. After two weeks, we measured the activity of the flow thru, flow

thru + stabilization protein, and flow thru + stabilization protein dialyzed. No phosphatase activity was observed in any of the samples.

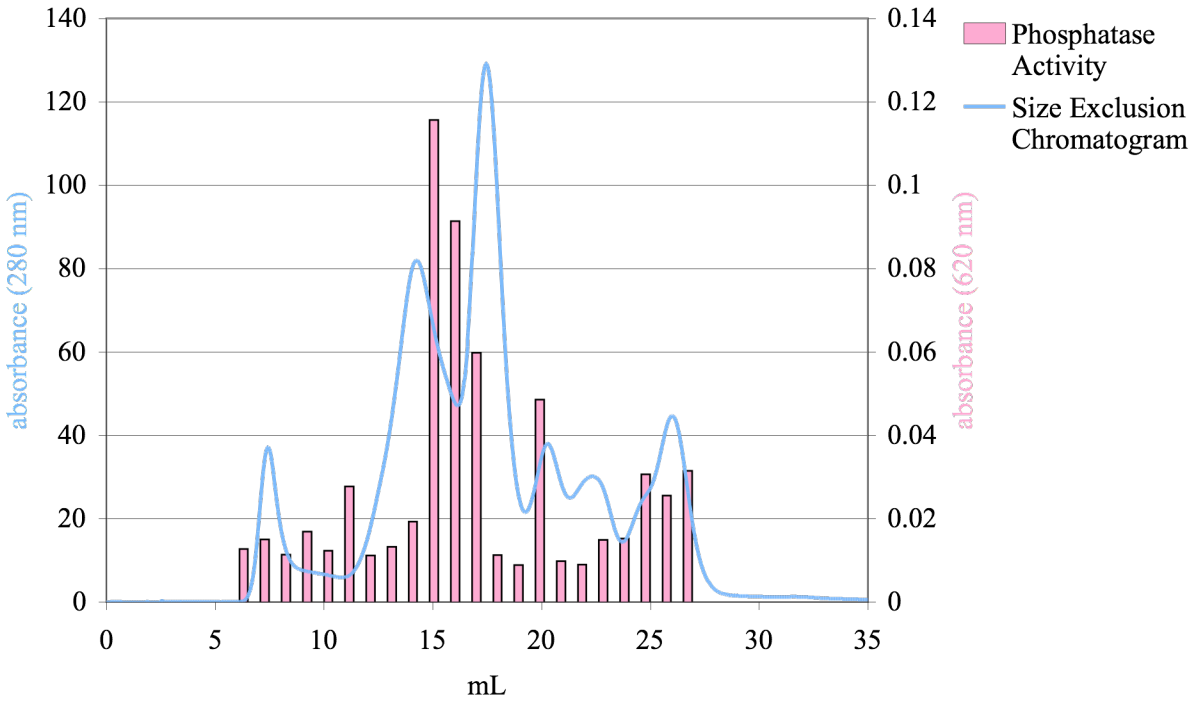
AM/MG assay – ammonium sulfate precipitation test

The old 40P sample coupled with the AM/MG assay led us to hypothesize that the phosphatase might actually precipitate out with ammonium sulfate at a lower concentration than previously thought. We prepared fractions from 0-20P, 20-30P, and 30-40P ammonium sulfate and tested them for activity. The phosphatase appeared to be active in the 0-20P ammonium sulfate fraction. Multiple 20P fractions were made thereafter because of the results obtained from this experiment, and attempts to purify the phosphatase from 20P fractions seemed promising because we were starting with a purer sample. In each case, we tested for enzymatic activity before proceeding to use a sample and attempt to further purify it.

AM/MG assay – 20P – size exclusion analysis

An FPLC equipped with the Superdex size exclusion column was used to separate a protein sample that had been precipitated with 20P ammonium sulfate. One mL fractions were collected and analyzed for phosphatase activity. Figure 8 shows the chromatogram with the corresponding phosphatase activity.

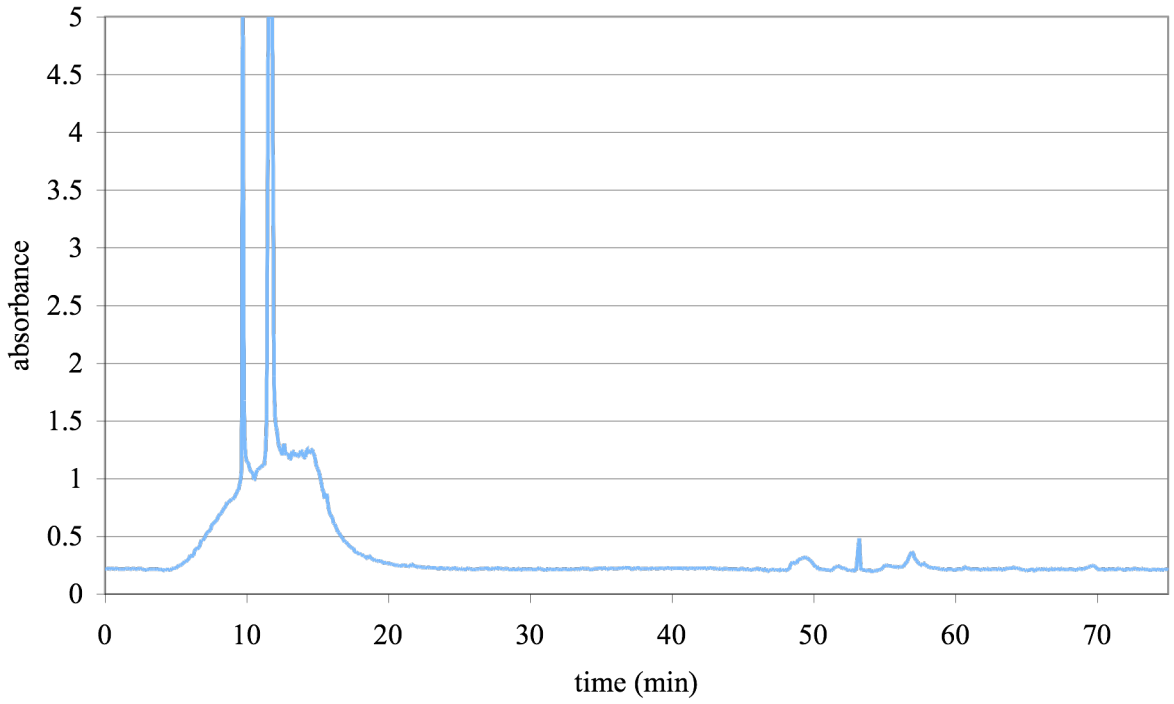
Figure 8. Measurement of phosphatase activity of various fractions following the separation of a 20P sample on the size exclusion column



AM/MG assay – CM column

The size exclusion column was run multiple times to collect enough sample to load it onto a CM column. The sample was eluted through the column using Buffer 4 at a pH of 7.0. The chromatogram did not look very promising, Figure 9, but each fraction was still tested for enzymatic activity. The presence of the phosphatase was not detected. This may have been the result of dilution to the sample or problems with the column.

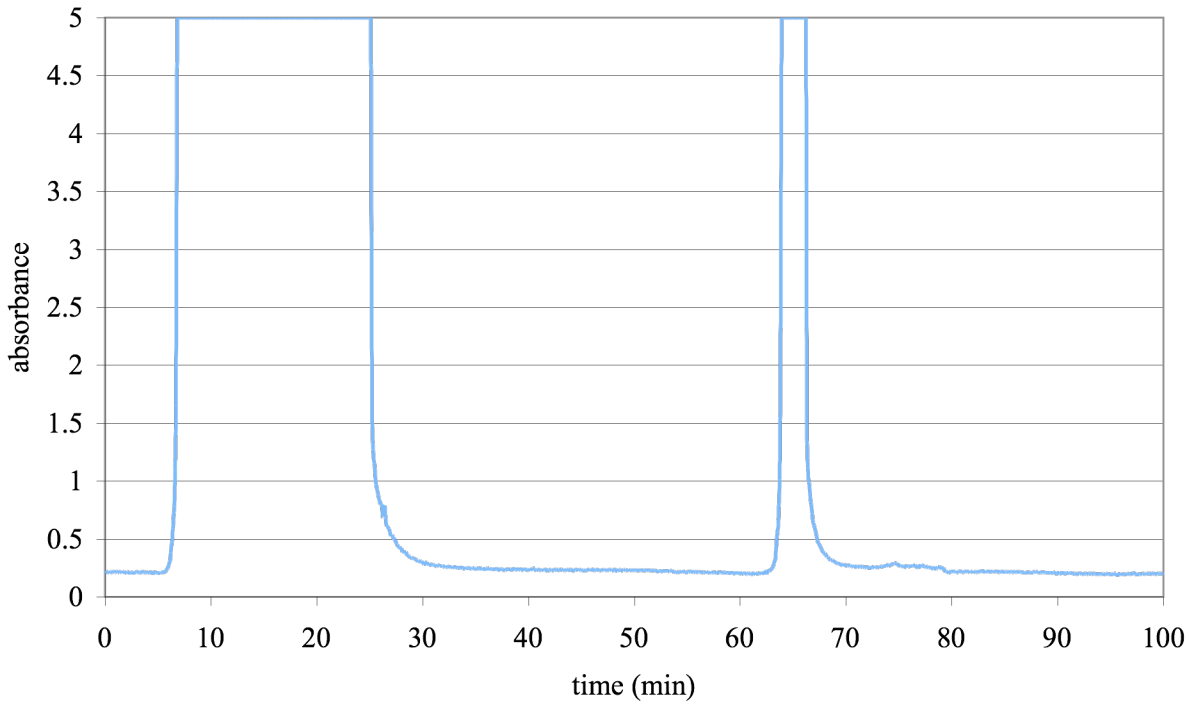
Figure 9. Chromatogram of a separation with the CM column following a separation of a 20P sample on the size exclusion column and lyophilization



AM/MG assay – High S column

Because we were unable to separate *glucose 1,6-bisphosphate* with the CM column, we attempted to use a High S column because it is a stronger cation exchange column. A 20P ammonium sulfate sample was prepared and loaded onto the column. The sample was eluted through the column using Buffer 4 at a pH of 7.0. A decent separation was achieved with this column, Figure 10, but no phosphatase activity was detected.

Figure 10. Chromatogram showing the separation of a 20P sample following separation on the High S ion exchange column



AM/MG assay – hydrophobicity columns

Because of the inability to predict which type of hydrophobicity column will retain a certain protein, six different types of hydrophobicity columns were loaded with a 20P sample. Each column was then eluted with a 10% ammonium sulfate solution to remove the sample and rinsed with buffer. The results from these experiments did not show any phosphatase activity.

AM/MG assay – rotofor cell

A 20P sample was loaded onto the rotofor cell. The results from this experiment were invalid because of cloudiness observed when the assay was added to the sample.

AM/MG assay – protein dilution test

We took great efforts to keep the samples concentrated so that we could perform more separation techniques for each sample, without having dilution problems. We therefore made dilutions of our 20P sample to determine if the increased protein concentration was causing the precipitation. Various dilutions ranging from 1:10 to 1:100 were made to test this hypothesis. Cloudiness still resulted in these samples. Cloudiness was common in several tissue samples. A lot of samples were prepared to try to avoid this problem. We could not determine the cause of the precipitation.

AM/MG assay – DEAE analysis at a higher pH

We experimented to determine whether we could get *glucose 1,6-bisphosphatase* to be retained by the DEAE column if we raised the pH of the buffer solution. A 20P sample was prepared in buffer 6 at a pH of 8.41. This sample was run on the DEAE. One of the retained peaks appeared to have active phosphatase activity. The active DEAE fractions were recombined and loaded onto the size exclusion column. The size exclusion fractions did not show any activity.

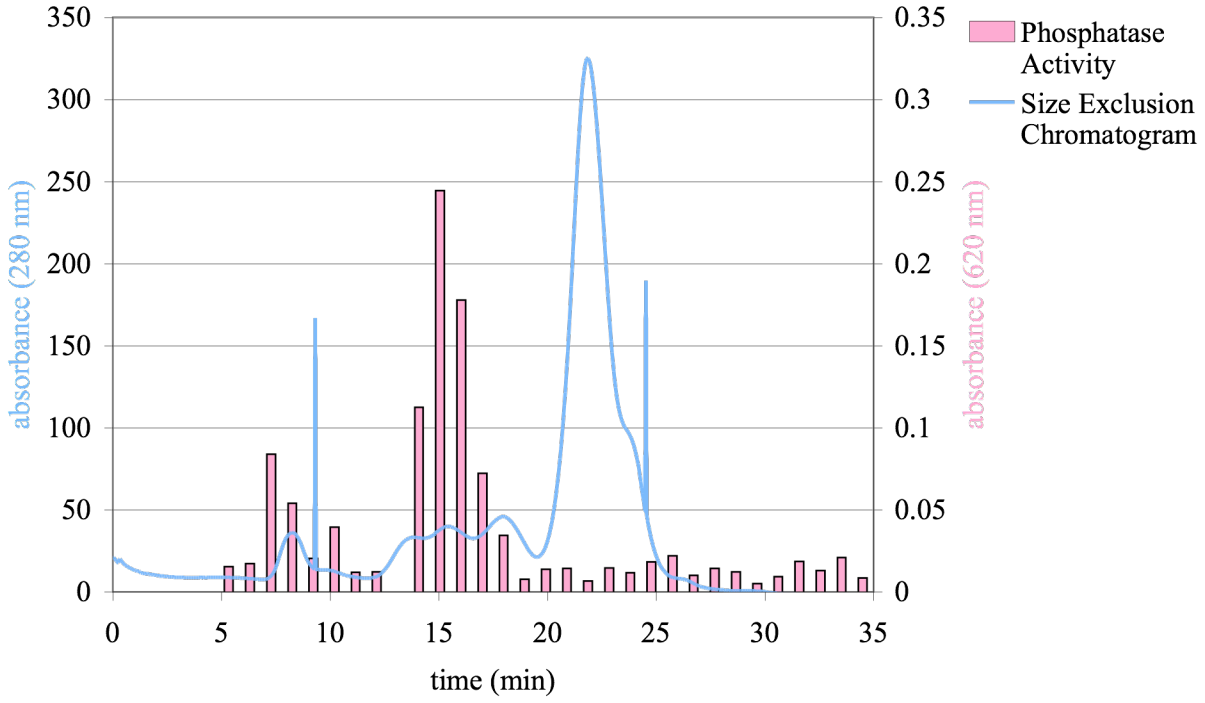
AM/MG assay – filter test

We attempted to use the 30, 50 and 100 kDa filters again with the new assay system. We thought that using the filters might make it so we could get our sample purer and concentrate it at the same time. However, the filters were extremely time intensive. Also there wasn't much benefit from these because the size exclusion column was giving reproducible results.

AM/MG assay – size exclusion – old 40P sample – stabilization analysis

The old 40P sample was separated on the size exclusion column and analyzed for phosphatase activity. Figure 11 shows the results of this experiment. Analysis showed the potential for additional phosphatase activity in addition to the normal peak. Fractions 7, 15, and 16 were each individually recombined with the other fractions to do a stabilization protein analysis. Fraction 14, which also showed considerable phosphatase activity, was lyophilized down (to concentrate the sample), digested with trypsin, and analyzed on the mass spectrometer.

Figure 11. Chromatogram showing the separation of the old 40P sample following separation on the size exclusion column



The stabilization analysis showed activity for all the fractions recombined with fraction 16 after two weeks. These results indicate that size exclusion is not a method that quickly denatures *glucose 1,6-bisphosphatase*.

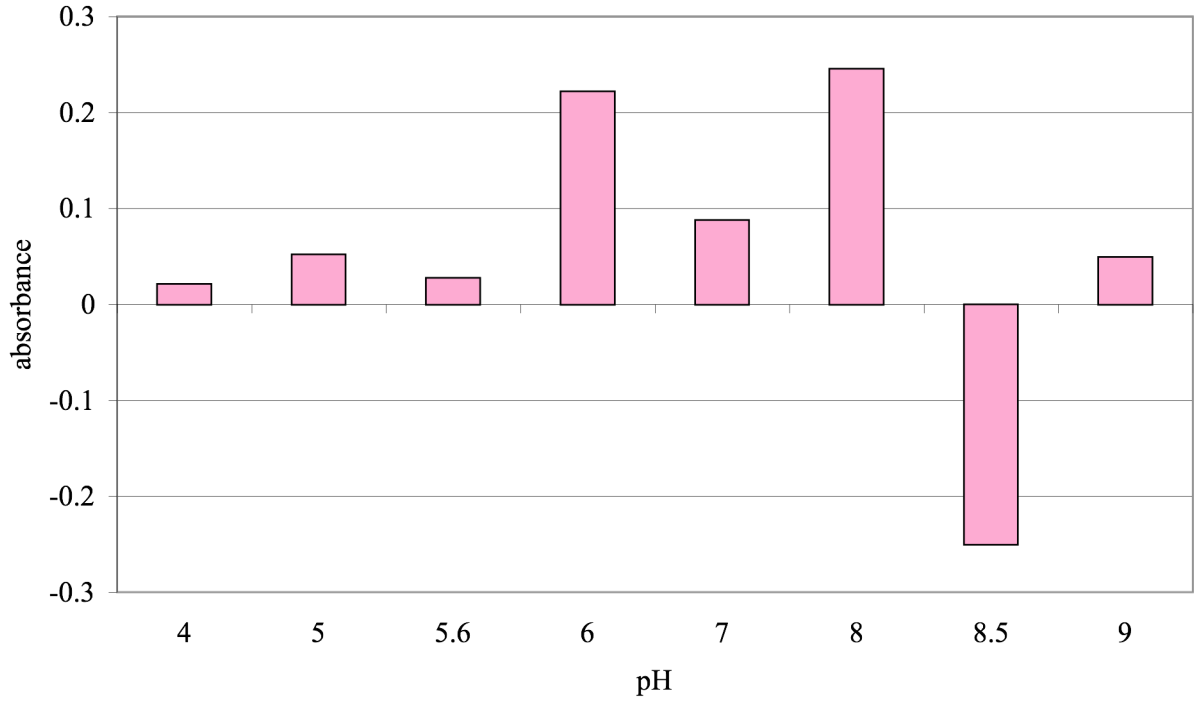
The stabilization analysis for the fractions recombined with fraction 7 showed a marked decrease in phosphatase activity for all the samples after two weeks. These results may indicate

the presence of a second *glucose 1,6-bisphosphatase* isoform that requires a stabilization protein, or a false positive from an earlier test.

AM/MG assay – old 40P sample – pH profile

We attempted to produce a pH profile of *glucose 1,6-bisphosphatase* using the old 40P sample. Various buffers ranging from pH 4.0-9.0 were analyzed to determine how they affected phosphatase activity. Figure 12 shows the results from this analysis.

Figure 12. pH profile of the old 40P sample showing the activity of *glucose 1,6-bisphosphatase* at various pH's



Despite repeating the experiment multiple times, we saw complications with the experiment run at pH 8.5 (consistent precipitation resulted with this sample, nullifying the effectiveness of the results). We also retested the sample run at a pH of 7 because it made the curve bimodal, but we repeatedly saw the same effect; the activity was not as high in this sample as with the samples run at pH 6 and 8. The results represented from this figure might be a result

of changing the buffer, or might indicate that there are two different isoforms that operate optimally at different pH's.

AM/MG assay – rotofor cell on raw lysate

We attempted to use the rotofor cell to separate the raw lysate without the commercial buffering system because it contained phosphates, rendering our assay useless. This experiment did not work, and it was determined that the commercial buffer system was required to get a decent separation.

AM/MG assay – DEAE on raw lysate

Ammonium sulfate precipitation had worked well to separate and purify protein samples. However, we wanted to test whether we could avoid some of the precipitation problems caused by the assay if this step were done later. A tissue sample was prepared and the raw lysate was loaded directly onto the DEAE before ammonium sulfate precipitation. The DEAE separated the sample, but sample/assay precipitation still caused issues rendering the results useless.

AM/MG assay – human erythrocytes

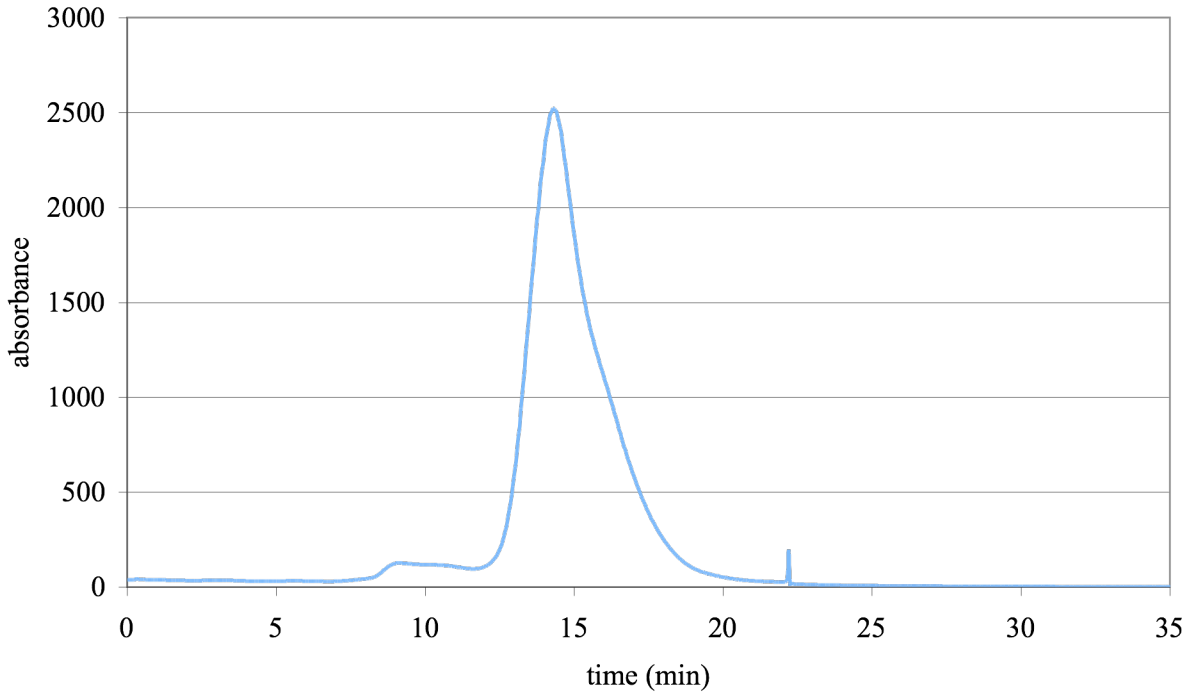
Blood samples were collected in heparin and EDTA. Both samples were spun to collect the erythrocytes. The samples were then washed, lysed, and buffer was added to prepare erythrocyte samples. Attempts to precipitate out only the hemoglobin with $ZnSO_4$ were unsuccessful. Because of the large concentration of hemoglobin in erythrocytes, $ZnSO_4$ precipitation appeared to pull out all other proteins from solution.

The EDTA sample was loaded onto the DEAE, but phosphatase activity was not observed after the separation.

The heparin sample was precipitated with ammonium sulfate to make a 40P solution. This sample was then loaded onto the size exclusion column. Figure 13 shows the results of this

test. Because of the high concentration of hemoglobin, it formed a very broad peak. This peak eluted over the *glucose 1,6-bisphosphatase* peak. These results confirmed an approximate mass for the phosphatase, but also showed the difficulty in separating out hemoglobin from the phosphatase. The sample also formed a precipitate with the assay, making it so the phosphatase could not be detected.

Figure 13. Chromatogram showing the separation of a red blood cell sample following separation on the size exclusion column



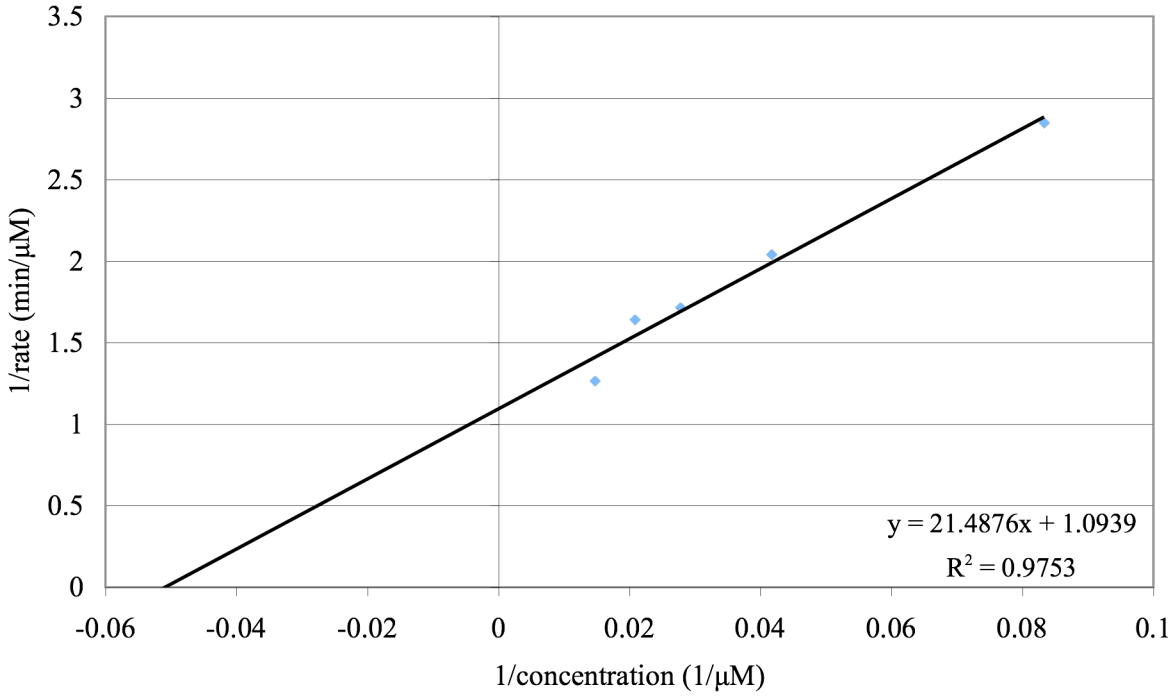
AM/MG assay – pork

Like liver, pork required more sample preparation than did the other samples, but we still attempted to use it as a source tissue. The sample was prepared similarly to other tissue samples, and run on the DEAE column. Analysis with pork revealed the same complications as observed with the other tissue samples, and consequently was abandoned as a source tissue.

AM/MG assay – K_m and V_{max}

K_m and V_{max} are common kinetic constants reported for enzymes. An advantage of these kinetic parameters is that it is not necessary to know the protein concentration to calculate these values, as it is with the turnover number. Figure 14 shows the Lineweaver-Burk plot from which the values of K_m and V_{max} were calculated. The calculated K_m and V_{max} for *glucose 1,6-bisphosphatase* were determined to be 19.6 μM and 0.91 μM/min, respectively.

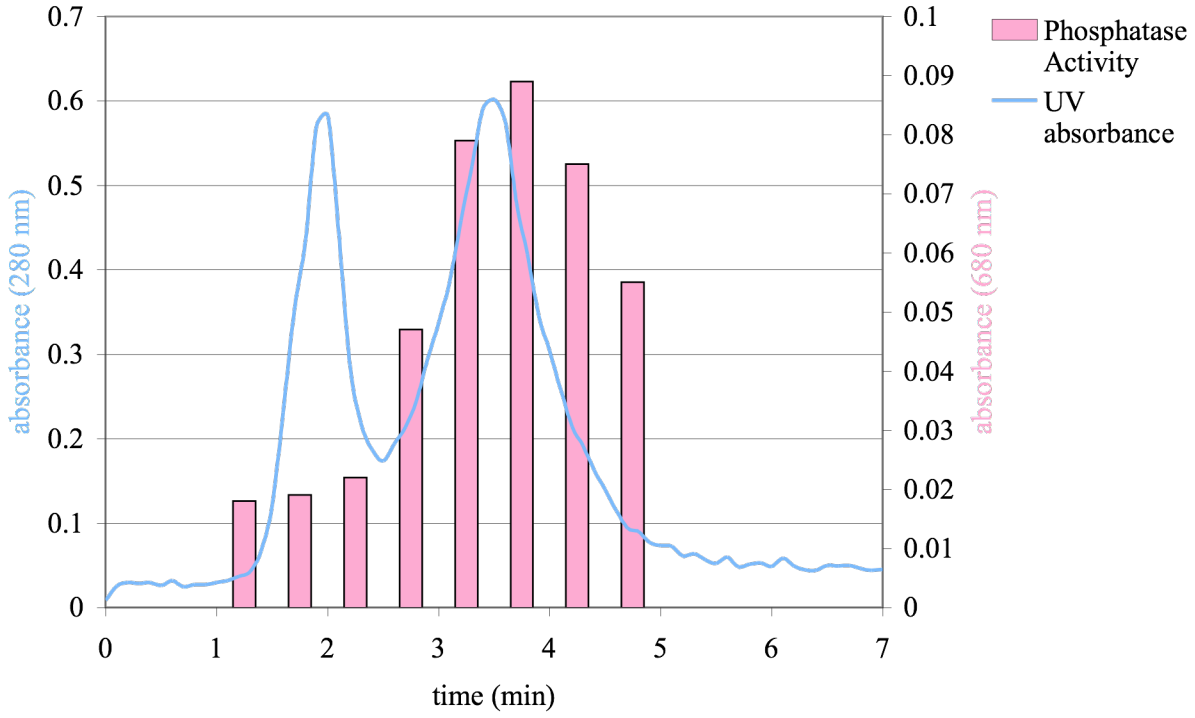
Figure 14. Lineweaver-Burk plot from which K_m and V_{max} of *glucose 1,6-bisphosphatase* were determined



AM/MG assay – CM column followed by size exclusion – old 40P sample

Old 40P sample was loaded onto a miniature CM column. Two peaks eluted off the column, as illustrated in Figure 15, before additional salt was added to remove proteins that stuck to the column. However, the additional salt did not reveal any other peaks. These results indicate that the old 40P samples still had some ions in solution, despite dialysis.

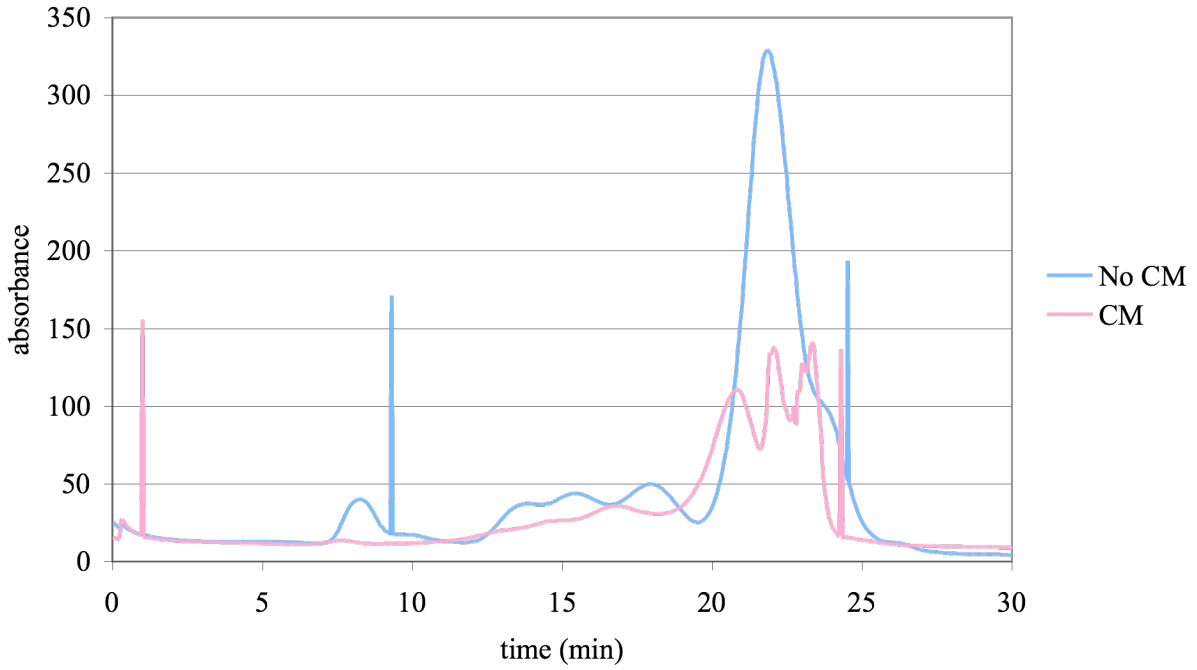
Figure 15. Chromatogram of the separation of the old 40P sample using the CM column



Fractions were collected in 0.5 mL volumes and analyzed for activity. *Glucose 1,6-bisphosphatase* activity was only observed in the second peak that eluted off the column.

Two of the fraction from the second peak were combined and loaded onto the size exclusion column. Figure 16 compares the chromatogram of the old 40P sample being separated on size exclusion with and without the CM separation.

Figure 16. Chromatograms comparing the separation of the old 40P sample following separation using the size exclusion column with and without prior separation on the CM ion exchange column



As with other size exclusion experiments, phosphatase activity was observed in fractions 14 and 15. These two fractions were combined, lyophilized, and a tryptic digest was performed to determine the proteins present in these fractions. The results from the tryptic digest only reported albumin and creatine kinase. It is expected that the relative concentration of the phosphatase was not large enough to be detected by mass spectrometry.

Phosphorous NMR – old 40P sample

As stated in the introduction, Hashimoto et al. *(5)* claimed that the reaction catalyzed by the muscle isoform of *glucose 1,6-bisphosphatase* formed Glc-1P, while Van Schaftingen et al. *(26)* observed that the brain isoform retained phosphoglucomutase activity, rendering it impossible to determine whether Glc-1P or Glc-6P was formed. We designed an experiment with phosphorous NMR to determine whether we could support the claim by Hashimoto et al.

Preliminary results indicated that Glc-1P, Glc-6P, and P_i were distinguishable by chemical shift. Glc-1,6P2 was analyzed later because of the cost of the chemical. Performing the Glc-1,6P2 experiment was only feasible if the other experiments showed promise. The analysis of Glc-1,6P2 revealed that it had approximately the same chemical shifts as Glc-1P and Glc-6P. This however was not a concern because we were interested in the ratio of one species to the other. Table 7 lists the chemical shifts observed for all the species analyzed. Whenever possible, experiments were run the same day to avoid fluctuations in the NMR and changes because the normal standards (TMS, $CDCl_3$) would not work for P-NMR. The chemical shifts in some samples moved as much as 1 ppm from day to day experiments.

Table 7. ^{31}P-NMR chemical shifts of compounds.

Compound	Chemical shift(s)
P_i	2-4[a]
Glc-1P	2.2-2.4
Glc-6P	4.4-4.5
Glc-1,6P2	2.2-2.4, 4.4-4.5

[a]chemical shift fluctuates significantly

With the exception of Glc-1,6P2, all the samples were prepared in D_2O. To prevent denaturization of the phosphatase, a 3:1 D_2O/tris buffer (100 mM, pH 7.5) solution was prepared for the Glc-1,6P2 sample, which would have the old 40P sample added to it after it had been analyzed on the NMR. The pulse angle for the NMR was set at 90 degrees and the relaxation delay was set at 25 seconds to ensure a proper scan. Figures 17 and 18 show the P-NMR spectra of the Glc-1,6P2 run and the Glc-1,6P2 with phosphatase run, respectively. Unfortunately, as illustrated in Figure 18, the P_i peak overlapped with the Glc-1P peak, rendering simple integration of peaks impossible. Visual analysis of the spectrum seems to indicate that the concentrations of Glc-1P and Glc-6P are equivalent. However, this observation cannot

definitively determine whether *glucose 1,6-bisphosphatase* has phosphoglucomutase properties because the old 40P sample was not entirely pure.

Figure 17. Phosphorous NMR of Glc-1,6P2.

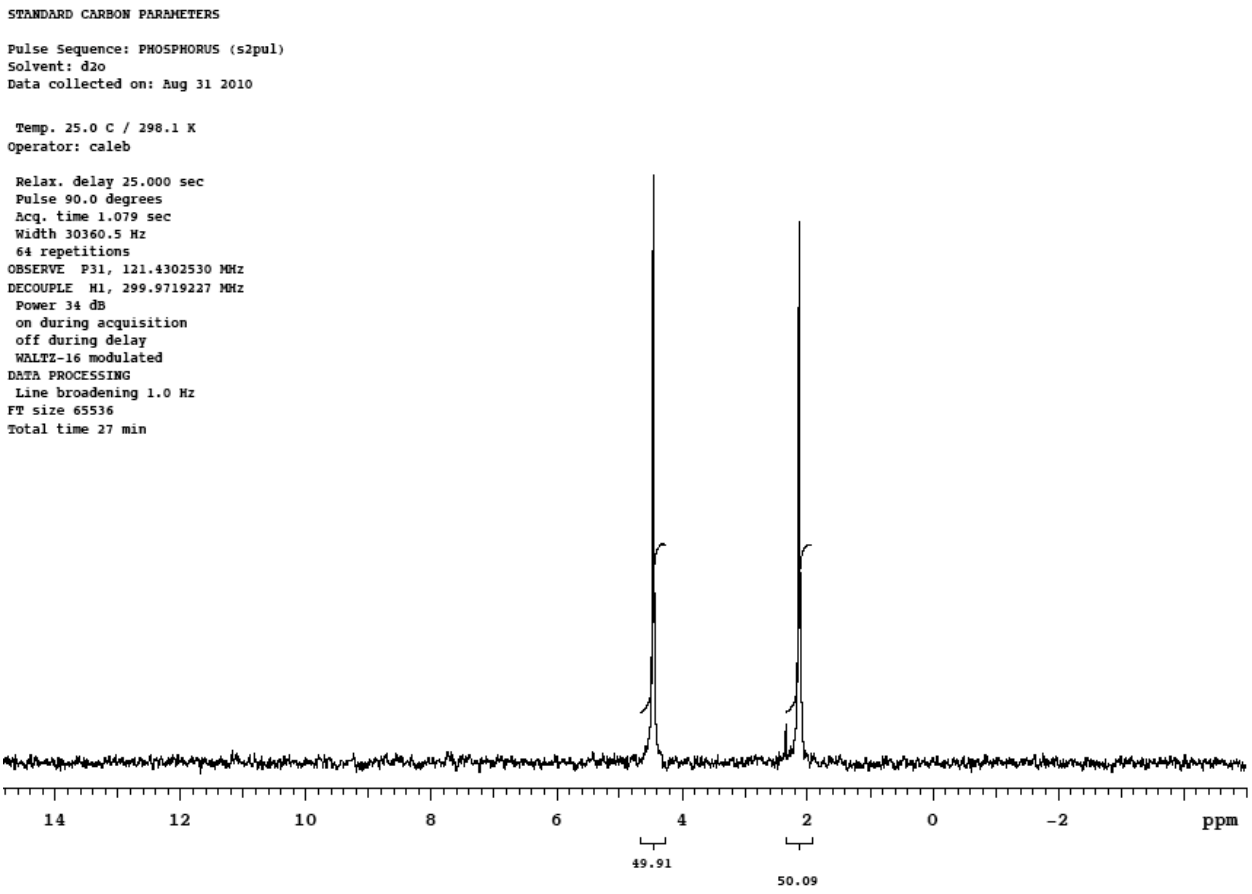
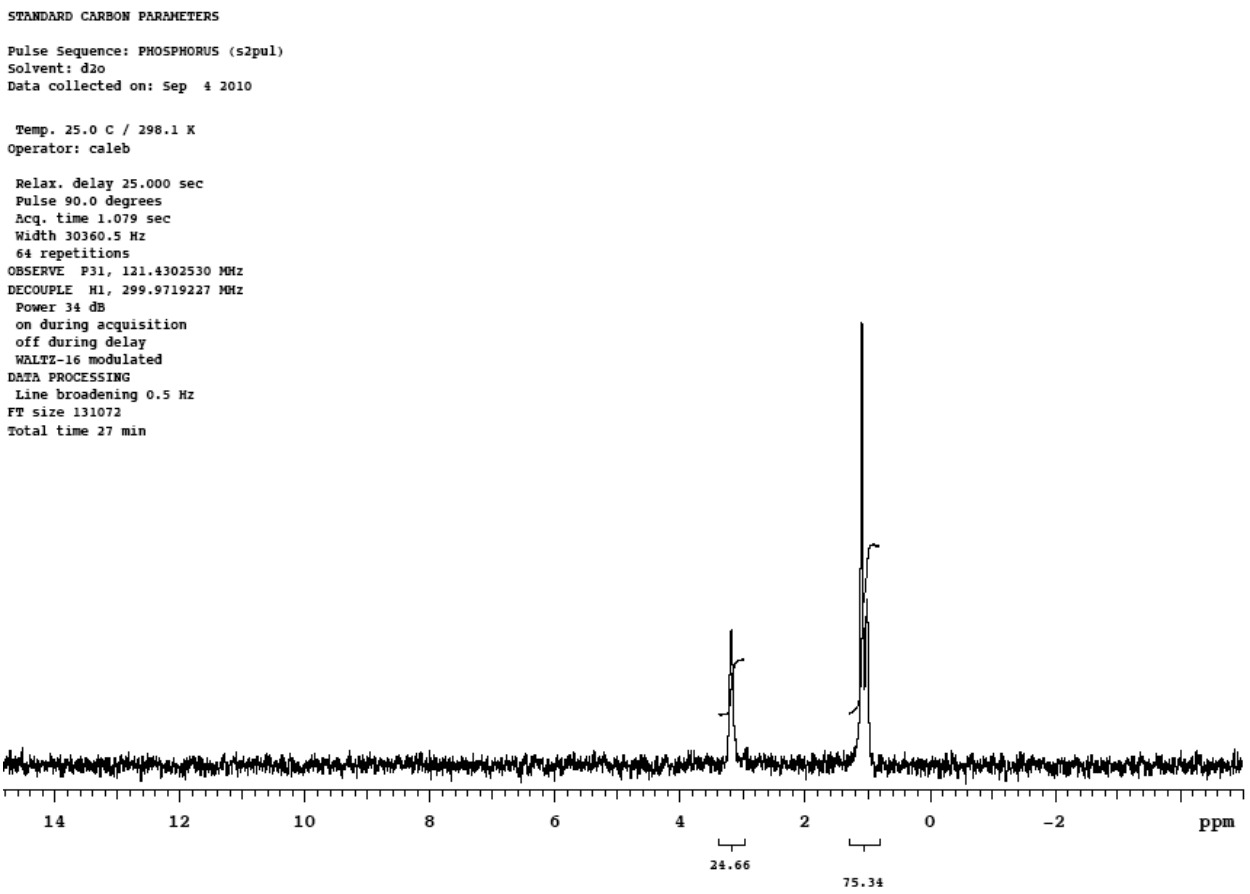


Figure 18. Phosphorous NMR of Glc-1,6P2 with *glucose 1,6-bisphosphatase*.



Summary of Results

Table 8 contains a summary of the results obtained from the experiments described in this section.

Table 8. Summary of Experiments and Results from Chapter 4.

Experiment title
Old 40P sample
Best protein sample prepared – showed longevity and did not cause precipitation
Commercial AM/MG assay
Identified the detection method of choice
AM/MG assay – DEAE – old 40P
DEAE destroyed phosphatase activity
AM/MG assay – DEAE – old 40P sample – stabilization analysis
Apparent stabilization protein observed – Figure 6 and Figure 7
Tryptic digest - DEAE – old 40P sample – stabilization analysis
Inconclusive results, only trypsin was detected
AM/MG assay – ammonium sulfate precipitation test
No phosphatase activity was observed
AM/MG assay – analytical size exclusion column
Sample too dilute to observe activity
AM/MG assay – C18 protein preparative column
Sample too dilute to observe activity
AM/MG assay – DEAE – C18
Sample too dilute to observe activity
AM/MG assay – activator and inhibitor tests
Over 100 compounds tested, only Frc-1,6P2 showed inhibitory properties
AM/MG assay - DEAE – old 40P sample – stabilization analysis with dialysis
No phosphatase activity was observed
AM/MG assay – ammonium sulfate precipitation test
Phosphatase precipitated out in 0-20P fraction
AM/MG assay – 20P – size exclusion analysis
Size exclusion separated sample – Figure 8
AM/MG assay – CM column
No phosphatase activity was observed – Figure 9
AM/MG assay – High S column
No phosphatase activity was observed – Figure 10
AM/MG assay – hydrophobicity columns
No phosphatase activity was observed
AM/MG assay – rotofor cell
Precipitation prevented analysis

AM/MG assay – protein dilution test

Protein dilution did not prevent precipitation with assay

AM/MG assay – DEAE analysis at a higher pH

Appeared to be retained by DEAE, fraction was inactive after size exclusion

AM/MG assay – filter test

Time intensive, no benefits over size exclusion

AM/MG assay – size exclusion – old 40P sample – stabilization analysis

Figure 11 shows size exclusion separation. Potentially two isoforms (various stability)

AM/MG assay – old 40P sample – pH profile

Bimodal optimum activities, potentially two isoforms – Figure 12

AM/MG assay – rotofor cell on raw lysate

Experiment did not work

AM/MG assay – DEAE on raw lysate

Tested order of operations, precipitation caused problems

AM/MG assay – human erythrocytes

Not a viable sample source, couldn't separate hemoglobin from sample – Figure 13 (size exclusion)

AM/MG assay – pork

Not a viable sample source

AM/MG assay – K_m and V_{max}

K_m and V_{max} were 19.6 μM and 0.91 μM/min, respectively. Lineweaver-Burk plot – Figure 14

AM/MG assay – CM column followed by size exclusion – old 40P sample

CM showed separation Figure 15, size exclusion was more pure Figure 16

Phosphorous NMR – old 40P sample

Unable to determine reaction catalyzed by phosphatase, Figures 17 and 18

Chapter 5

Discussion and Future Work

The main goal of this project was not achieved, to isolate *glucose 1,6-bisphosphatase*, but much was learned about the enzyme in the journey. Table 8 summarizes the experiments that showed promise. The project had many snags, which slowed progress. This section is designed to discuss the problems we faced, and state how we were able to overcome them. It will also summarize the advantages and disadvantages of the separation techniques used with regards to this project.

Detection method

A considerable amount of time was spent identifying a method to detect the enzymatic reaction carried out by *glucose 1,6-bisphosphatase*. Assays giving false positives made it difficult to distinguish their insufficiencies with this project. Eventually we settled on the AM/MG assay. This tested for P_i and seemed to give the most reliable results. Most of our progress came about when this assay was being used.

The AM/MG assay, however, was not without its pitfalls. We never could determine what caused the precipitation when the assay was combined with a sample. Many samples had to be discarded because of this problem. The only sample that did not show signs of precipitation was the old 40P sample. Despite efforts to recreate this sample, it could not be mimicked. Extreme care was taken to perform trial runs with other samples before the old 40P sample was analyzed.

Since the old 40P sample could not be reproduced, many skeptics will wonder whether this sample was even good to begin with or if *glucose 1,6-bisphosphatase* even exists. There was a distinguishable color change, evident by visual analysis, after the old 40P sample had been separated. Use of the spectrophotometer was often not even needed when the sample was being

analyzed (of course it still was used to confirm phosphatase activity). This sample confirmed the presence of a phosphatase.

Buffers

Buffer 5 ended up as the buffer of choice for this project. It was simple to prepare and user friendly. Some of the other buffers were more difficult to prepare because of solubility issues. The old 40P sample was prepared in buffer 4, but later experiments used buffer 5 as the mobile phase and no loss of activity was observed.

Ammonium sulfate precipitation

Conflicting results between early experiments and later experiments showed phosphatase activity in different ammonium sulfate fractions. Later results revealed that *glucose 1,6-bisphosphatase* precipitated out in the 20P fraction. Although precipitation problems caused some challenges, the fact that size exclusion separated the 20P sample and showed phosphatase activity was very encouraging.

Spin filters – ultrafiltration

Spin filters had no advantage over size exclusion as a separation technique and would not be recommended as a purification technique for the purifying of *glucose 1,6-bisphosphatase*.

Tube gel – size exclusion apparatus

The tube gel had no advantage over size exclusion as a separation technique and would not be recommended as a purification technique for the purifying of *glucose 1,6-bisphosphatase*. Additionally, the tube gel diluted samples too much to observe phosphatase activity. This was not a viable separation technique.

Gel electrophoresis

The technique was useful in determining the purity of the sample and progress made towards the isolation of the phosphatase. However, it was not useful large-scale separations.

Rotofor cell – isoelectric focusing

The rotofor cell was not a useful separation technique. The buffer used to make the pH gradient contained phosphate and prevented the assay from working correctly. Also, because of the volume of the sample, dialysis was not a viable technique to remove the additional phosphatase.

Size exclusion

The most useful and reliable separation technique that we performed on samples was with the preparatory size exclusion column. Phosphatase activity was consistently seen in the same fractions, whether it was from the old 40P sample or a 20P sample. The major setback with this technique is that only 0.5 mL of sample can be loaded onto the column at a time and each run takes over an hour. Therefore this separation technique serves for a final separation technique and realistically cannot be used as a large-scale separation technique.

DEAE – anion exchange chromatography

Initially, the DEAE appeared to be a fantastic separation technique. However, it was later observed that enzymatic activity disappeared after using this technique. Therefore, it was determined that this technique needed either be abandoned or that samples must undergo additional purification techniques soon after using the DEAE column. However, this technique raised the question as to whether or not a stabilization protein is the cause of the difficulties in isolating the phosphatase.

CM – cation exchange chromatography

Later results with the CM column were favorable as to its use as a separation technique. The column we had was a smaller column and did not allow for large-scale separations. Because the benefits of this technique were observed near the end of my time in the lab, I did not have the opportunity to make a larger column. Future work could be dedicated to using this column to aid to purify the phosphatase.

High S – cation exchange chromatography

As the High S column is similar to the CM column, exploring its use as a purification technique would also be advantageous. However, the experiments with the small column did not reveal phosphatase activity. We were not able to determine if that was because of an issue with the sample or the effects of the column.

Hydrophobicity columns

None of the hydrophobicity columns we tested appeared to retain the phosphatase. They were all miniature columns, however, and may actually serve to partially purify a sample if larger columns were analyzed.

$ZnSO_4$

The first experiments performed showed that hemoglobin was more of a problem than in later experiments. It was therefore determined that using $ZnSO_4$ was not a necessary separation technique.

Reverse phase chromatography (C18)

No matter what we attempted to do to samples to keep them concentrated (minimally dilute samples when resusupending them after ammonium sulfate precipitation, lyophilization, or ultrafiltration), separation on the C18 column diluted the sample too much to observe

phosphatase activity. This technique was not useful in helping to purify *glucose 1,6-bisphosphatase*.

Glucosamine affinity column

The glucosamine affinity column showed no signs of retaining any proteins. Despite the lack of protein absorbance measured on the UV detector, fractions were still tested for phosphatase activity. However, no phosphatase activity was observed. This column was not useful to help purify *glucose 1,6-bisphosphatase*.

Future work

Additional work needs to be done to isolate *glucose 1,6-bisphosphatase*. Overcoming the precipitation issues, and being able to consistently prepare viable samples is a major step that needs to be accomplished if the phosphatase is to be separated. Also, it needs to be determined if there is in fact a stabilization protein that prevents the phosphatase from losing activity. If there is, it should be added back to the sample after each separation technique.

To date, the best purification method we've developed to isolate the phosphatase is with the use of the preparatory size exclusion column. However, it is unrealistic to do large-scale separations with this technique because of the time that would be involved to collect enough of the sample to take it on to the next purification technique, which may or may not work.

Supplemental Material

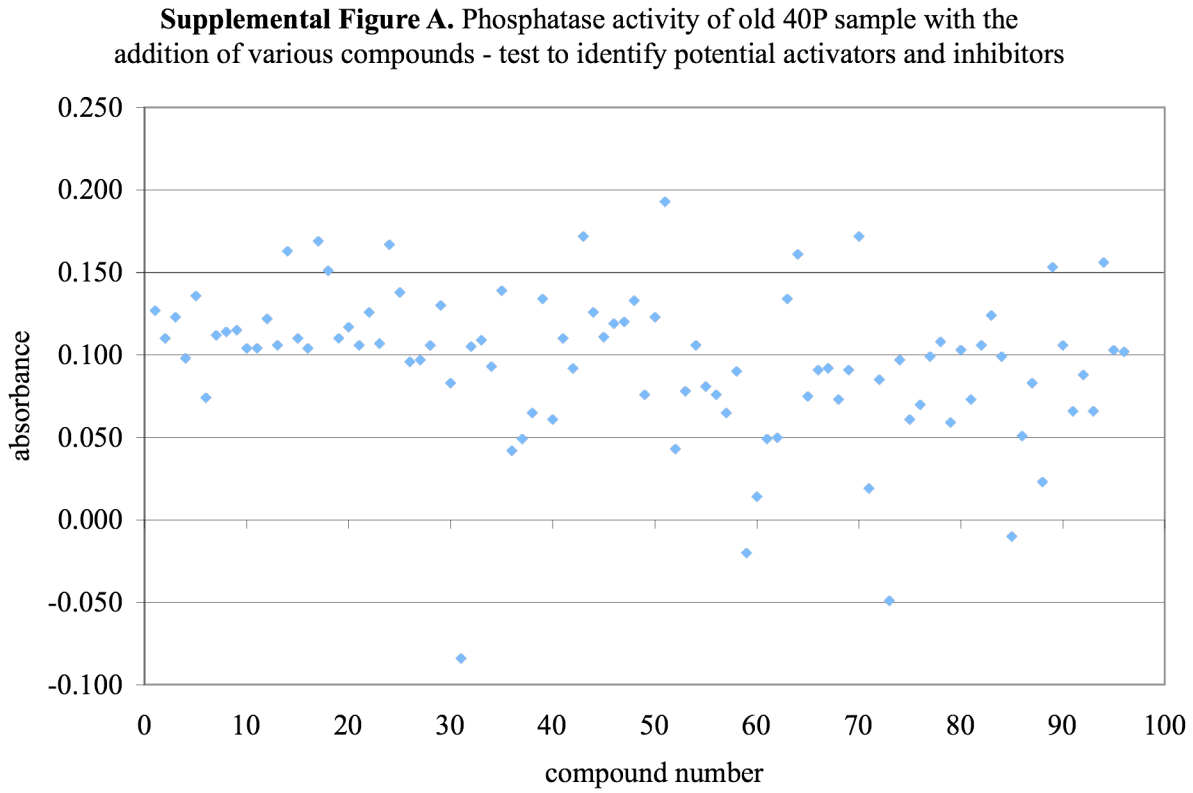
Supplemental Table A. List of compounds tested as activators and inhibitors of *glucose 1,6-bisphosphatase*.

compound #	compound	absorbance
1	acenaphthene	0.127
2	acenaphthenequinon	0.110
3	1-adamantanol	0.123
4	2-acetylfluorene	0.098
5	acetylcholine iodide	0.136
6	4-acetamidophenol	0.074
7	adipic acid	0.112
8	2-allylphenol	0.114
9	4-acetylbenzonitrile	0.115
10	adamantane	0.104
11	DL aspartic acid	0.104
12	aminoacetic acid	0.122
13	4-aminoacetophenone	0.106
14	p-aminobenzoic acid	0.163
15	4-aminosalicylic acid	0.110
16	2-amino-4-cholorphenol	0.104
17	3-aminorhodanine	0.169
18	4-amino-3-nitrobiphenyl	0.151
19	β-alanine	0.110
20	9-anthracenecarboxylic acid	0.117
21	4-aminoantipyrine	0.106
22	2-amino-5-nitrophenol	0.126
23	9-anthraldehyde	0.107
24	anthranilic acid	0.167
25	4-aminoantipyrine	0.138
26	3-aminobenzoic acid	0.096
27	p-aminophenol	0.097
28	L-+-arabinose	0.106
29	anthrone	0.130
30	L-ascorbic acid	0.083
31	L-arginine	-0.084
32	L-asparagine	0.105
33	antipyrine	0.109
34	o-arsanilic acid	0.093
35	azoxybenzene	0.139
36	azobenzene	0.042
37	behenic acid	0.049
38	5,6-benzoquinoline	0.065
39	benzoin oxime	0.134

40	benzanilide	0.061
41	1,4-benzoquinone	0.110
42	9-bromoanthracene	0.092
43	benzotriazole	0.172
44	2,2-bipyridine	0.126
45	4,4-biphenyldicarboxylic acid	0.111
46	benzimidazole	0.119
47	benzhydrol	0.120
48	biphenyl	0.133
49	1-bromo-4-iodobenzene	0.076
50	n-bromo-succinimide	0.123
51	p-bromobenzoic acid	0.193
52	1-bromoadamantane	0.043
53	(+)-bromosuccinic acid	0.078
54	p-bromobenzenesulfonyl chloride	0.106
55	4-bromopyridine hydrochloride	0.081
56	4-bromothioanisole	0.076
57	5-bromosalicylaldehyde	0.065
58	α-bromo-p-tolunitrile	0.090
59	4-tert-butyl-cyclohexanone	-0.020
60	tert-butyl carbazate	0.014
61	catechol	0.049
62	4-tertbutylphenol	0.050
63	1S-(+)-10-camphorsulfonic acid	0.134
64	carbazole	0.161
65	p-bromoaniline	0.075
66	4-carboxybenzaledhyde	0.091
67	coumarin	0.092
68	4-chlorobenzaldehyde	0.073
69	p-cholrphenacyl bromide	0.091
70	caffeine	0.172
71	cyanoguanidine	0.019
72	ceric sulfate	0.085
73	cis-1,2-cyclohexanedicarboxylic anhydride	-0.049
74	choloracetophenone	0.097
75	cholesterol	0.061
76	1-chloro-4-nitrobenzene	0.070
77	monocholoracetic acid	0.099
78	4-chlorobenzyl alcohol	0.108
79	citric acid	0.059
80	3-chloropropionic acid	0.103
81	4-chloro-2-nitrobenzyl	0.073
82	1-chloro-2,4-dinitrobenzene	0.106

83	o-chlorocinnamic acid	0.124
84	chlorotetracycline HCL	0.099
85	2-chloroquinoline	-0.010
86	4-chloro-3,5-dimethylphenol	0.051
87	2,6-di-tert-butyl-4-methylphenol	0.083
88	3,4-dimethyl-5-thiazolium iodide	0.023
89	4-chloroacetanilide	0.153
90	creatinine	0.106
91	chrysophenine	0.066
92	2,4-dinitrophenol	0.088
93	4-cyanobenzaldehyde	0.066
94	4-cyanophenol	0.156
95	L-cysteine HCL	0.103
96	cis-4-cyclohexene-1,2-dicarboxylic acid	0.102

Supplemental Figure A. Results of activator and inhibitor testing.



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