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Expression and Purification of Human Lysosomal β -galactosidase from *Pichia Pastoris*

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**EXPRESSION AND PURIFICATION OF HUMAN LYSOSOMAL
 β -GALACTOSIDASE FROM *PICHA PASTORIS***

A Thesis Presented

by

SARAH TARULLO

Submitted to the Graduate school of the
University of Massachusetts Amherst in partial fulfillment of the requirements for the
degree of

MASTER OF SCIENCE

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ABSTRACT

EXPRESSION AND PURIFICATION OF HUMAN LYSOSOMAL β -GALACTOSIDASE FROM *PICHTIA PASTORIS*

SEPTEMBER 2014

SARAH TARULLO, B.S UNIVERSITY OF MASSACHUSETTS AMHERST

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Lysosomal storage diseases are genetically inherited diseases caused by the dysfunction of lysosomal enzymes. In a normal cell, lysosomal enzymes cleave specific macromolecules as they are transported to the lysosome. However, in diseased cells, these lysosomal enzymes are either absent or malfunctioning, causing macromolecular substrates to accumulate, becoming toxic to the cell. Over fifty lysosomal storage diseases have been identified, collectively occurring in one out of 7,700 live births. We investigated the lysosomal enzyme β -galactosidase (β -gal). In order to study the biochemistry and enzymology of this protein a robust expression system was needed. The *GLB1* gene has been inserted into *Pichia pastoris* creating high protein expressing cell lines. The result of this work will yield a high expression system for β -gal, which can then be subjected to structural and biochemical studies.

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CHAPTER 1

INTRODUCTION

Lysosomal Storage Diseases

Lysosomal storage diseases are human genetic disorders caused by the malfunction of lysosomal enzymes. In a normal cell, these enzymes are translated off of the ribosomes and translocated into the endoplasmic reticulum (ER). In the ER, these proteins are modified and interact with macromolecular chaperones that ultimately result in properly folded protein. From the ER, lysosomal proteins are then transported to the Golgi apparatus, where they are further post-translationally modified and transported to the lysosome. Once in the lysosome, lysosomal enzymes are then responsible for the metabolite processing and degradation of a wide variety of substrates. In humans, a

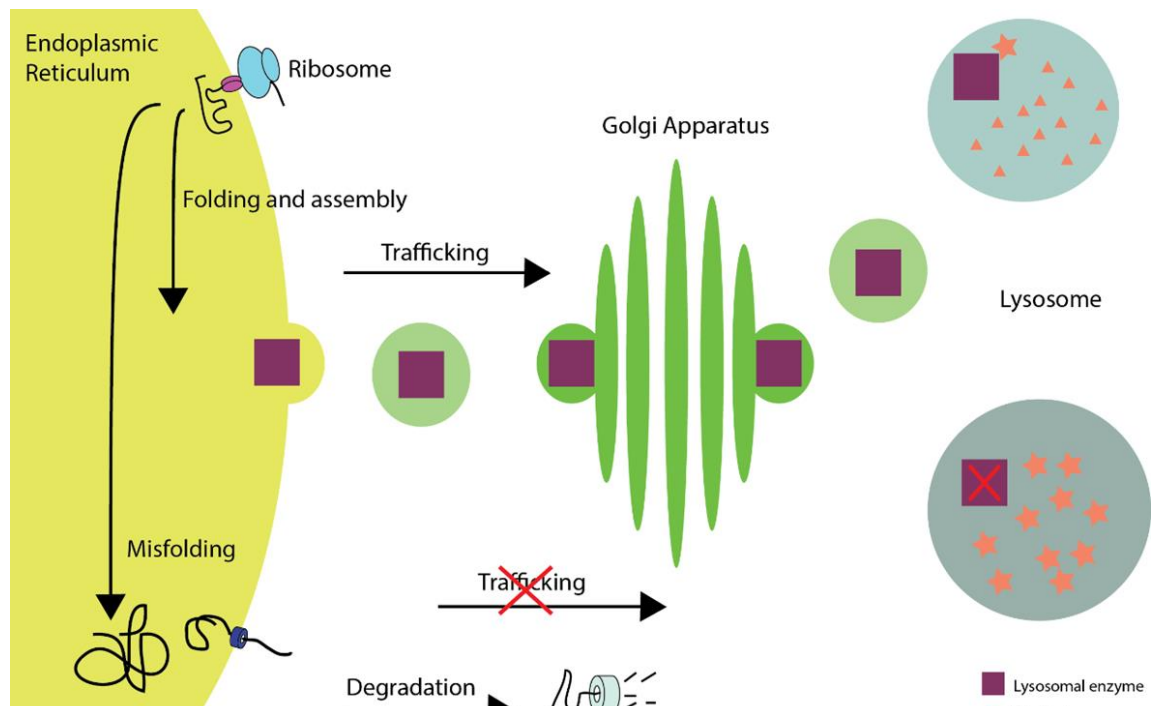


Figure 1. Cartoon of lysosomal storage disease mechanism. Top depicts the normal trafficking of lysosomal proteins to the lysosome. Polypeptide is translocated into the ribosome where it is properly folded and assembled. It is then trafficked to the Golgi Apparatus and finally to the lysosome where it can then degrade substrate. The bottom depicts what occurs in lysosomal storage disease, where protein is either not made or misfolded, resulting in degradation via the proteasome. Lysosomal proteins never reach the lysosome, causing a build-up of substrate, resulting in disease.

deficiency of a single lysosomal enzyme leads to substrate accumulation, which becomes toxic to the cell and causes lysosomes to swell. A cartoon of this is shown in Figure 1. These metabolic defects are collectively known as lysosomal storage diseases. Currently over 50 lysosomal storage diseases have been discovered, collectively affecting 1 in 7,700 live births (Staretz-Chacham, et al. 2009). Although there are some caused-based treatments for lysosomal storage diseases, many therapies are only focused on the alleviation of symptoms.

Caused-based treatment options for lysosomal storage diseases include substrate reduction therapy, stem cell therapy, gene therapy, enzyme replacement therapy, and pharmacological chaperone therapy (Lim-Melia, et al. 2009). Currently, enzyme replacement therapy is the most common Federal Drug Administration (FDA) approved treatment for lysosomal storage disease. It is approved for seven lysosomal storage diseases: Fabry disease, Gaucher disease, Pompe disease and Mucopolysaccharidosis Types I, II, IVA and VI (Lim-Melia, et al. 2009, BioMarin, 2014). However, enzyme replacement therapy is very expensive, costing patients \$200,000-\$300,000 annually (Beutler, 2006). These patients must receive intravenous infusions of recombinant enzymes on a weekly or bi-weekly basis (Lim-Melia, et al. 2009).

An alternative, less invasive treatment is pharmacological chaperone therapy. This approach uses small molecules that are competitive inhibitors of specific lysosomal enzymes. Traditional pharmacological chaperones bind to the active site of mutant lysosomal enzymes in the ER, causing a shift in equilibrium of the lysosomal enzyme toward the folded protein state (Fan, 2003). The folded protein can then reach the lysosome and degrade substrate. Although not an option for all genotypes,

pharmacological chaperones appear to be a promising treatment option for lysosomal storage diseases. Currently deoxygalactonojirimycin (DGJ), a small molecule pharmacological chaperone, is in Phase III clinical trials to treat Fabry Disease, a metabolic disease caused by defects in lysosomal α -galactosidase (Benjamin, et al. 2009). Another small molecule tested in clinical trials is isofagomine or Plicera™. Although found to have *in vitro* effects (Chang, et al. 2006), clinical trials proved less promising (Amicus, 2009).

Human Lysosomal β -galactosidase (GLB1)

Human lysosomal β -galactosidase is an enzyme encoded on chromosome 3 by the *GLB1* gene (Suzuki, et al. 1995). This enzyme is responsible for the cleavage of terminal β -linked galactose residues from glycoproteins, sphingolipids, keratan sulfate, and other glycoconjugates (Suzuki, et al. 1995). Previous studies have shown the reaction mechanism of GLB1 to be a double displacement reaction (McCarter, et al. 1997 Ohto, et al. 2012). This reaction requires two carboxylic groups, one to act as a catalytic nucleophile, the other as an acid/base catalyst. (Koshland and Stein, 1954). It has been



Figure 2. Processing of GLB1. Monomeric GLB1 highlighting protein cleavage to the mature form with galactose soaked into the active site. N-terminal region is shown in purple; the C-terminal region shown in pale yellow. The precursor form of GLB1 is cleaved into this mature form, where the C-terminal fragment remains associated with the N-terminal region. Figure generated using PBID: 3THC.

determined that in GLB1 the catalytic nucleophile is E268 and the acid/base catalysis is E188 (McCarter, et al 1997). GLB1 is synthesized as an 85 kDa protein, and when phosphorylated results in an 88 kDa precursor protein (Callahan, 1999). This precursor is then processed into the mature 64 kDa protein via cleavage of the C-terminal end which remains associated with the mature protein as shown in Figure 2 (Callahan, 1999, van der Spoel, et al. 2000). Previous studies have shown that the precursor enzyme has normal activity (Zhang, et al. 1994), but it is not known whether the precursor has activity against native substrate (Callahan, 1999). GLB1 is trafficked to the lysosome in a multi-enzyme complex consisting of two other lysosomal enzymes, protective protein/Cathepsin A (PPCA) and neuramidase I (NEU1) (van der Spoel, et al. 2000). However some patients with a PPCA deficiency retain some GLB1 activity, thus suggesting an alternative trafficking pathway for GLB1 outside this multi-enzyme complex (Hoogeveen, et al. 1983).

The endogenous GLB1 enzyme has been purified from liver cells (Norden, et al. 1974), human fibroblasts (Fuyura, et al. 2008), and human placenta (Lo, et al. 1979). Recombinant GLB1 enzyme has also been expressed and purified from eukaryotic cells such as Chinese Hamster Ovary (CHO), (Zhang, et al. 1994) and *Pichia pastoris* yeast cells (Ohto, et al. 2012). Enzyme activity has been reported from both purified recombinant enzyme and from cells of G_{M1} -gangliosidosis patients. In order for stored substrate (G_{M1} -ganglioside and/or keratan sulfate) to be cleared, an estimated 10% of normal GLB1 activity is needed (Suzuki, 2006). Recombinant GLB1 purified from CHO cells showed optimal enzymatic activity at pH 4.3, with a reported K_M and V_{max} of 0.29 mM and 989 mmol/h/mg respectively, using the fluorescent synthetic substrate 4-

methylumbelliferyl- β -D-galactoside (4MU- β -gal) (Zhang, et al. 1994). Using 2, 4-dinitrophenyl- β -D-galactoside, a colorimetric synthetic substrate, a K_M of 0.73 mM and V_{max} of 888 mmol/hr/mg were reported (Zhang, et al. 1994). GLB1 activity from skin fibroblasts from patients with infantile G_{M1} -gangliosidosis was less than 1% of control fibroblasts (Hoogeveen, et al. 1984). In fibroblasts from patients with Morquio B Disease and adult G_{M1} - gangliosidosis activity was measured to be 4-9% of normal fibroblasts.

The crystal structure of the GLB1 dimer was published in 2012 by Ohto et al. (Figure 3). They reported the protein crystal structure of recombinant GLB1 from yeast cells, *Pichia pastoris*. GLB1 had been purified, deglycosylated, and trypsinized to mature GLB1 with galactose (PBID: 3HTC) and DGJ (PBID: 3THD) soaked into the active site. It was reported that GLB1 is a dimer at pH 4.5 (Ohto, et al. 2012), although previous studies have reported that it is a monomer at neutral pH (Norden, et al. 1974). The monomeric structure has seven glycosylation sites in addition to three distinct domains:

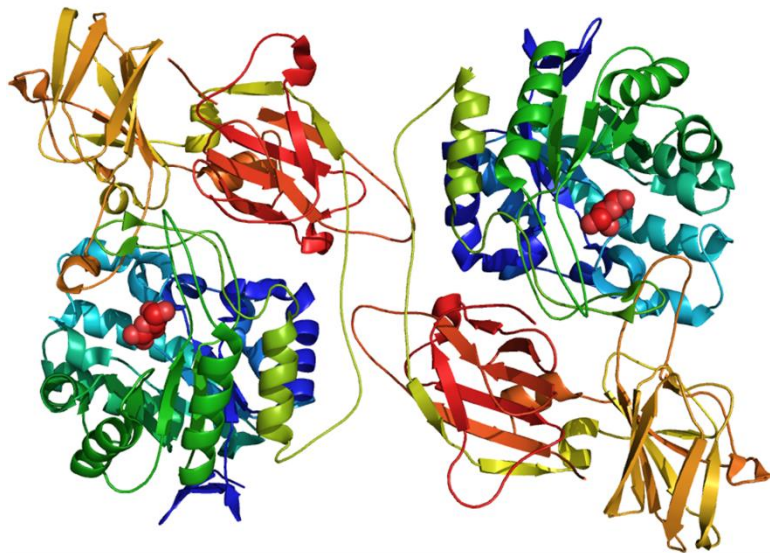


Figure 3. Dimer crystal structure of GLB1. The crystal structure of GLB1 was solved to 1.8 angstrom resolution in the space group $P2_1$ shown here as a dimer and with galactose (red spheres) soaked into the active site. Figure generated using PBID: 3THC.

the catalytic TIM barrel domain and two β -domains (Ohto, et al. 2012). The GLB1 active site is formed at the C-terminal end of the β -strands in the eight-stranded α/β barrel. The data obtained from this crystal structure and its analysis is crucial in attempts for future structure-based drug design of inhibitors and possible pharmacological chaperone candidates.

G_{M1}-gangliosidosis and Morquio B Disease

Over 100 mutations have been discovered in the human GLB1 gene, leading to two distinct lysosomal storage diseases: G_{M1}-gangliosidosis and Morquio B disease (Brunetti-Pierri, et al. 2008). G_{M1}-gangliosidosis is an autosomal recessive disease caused by the accumulation of the glycolipid G_{M1}-ganglioside in the central nervous system (CNS) (Sinigerska, et al. 2006). Patients with this disease display symptoms of intellectual disability, seizures, liver and spleen enlargement, and neurodegeneration (Brunetti-Pierri, et al. 2008). G_{M1}-gangliosidosis affects 1 in 100,000-200,000 newborns

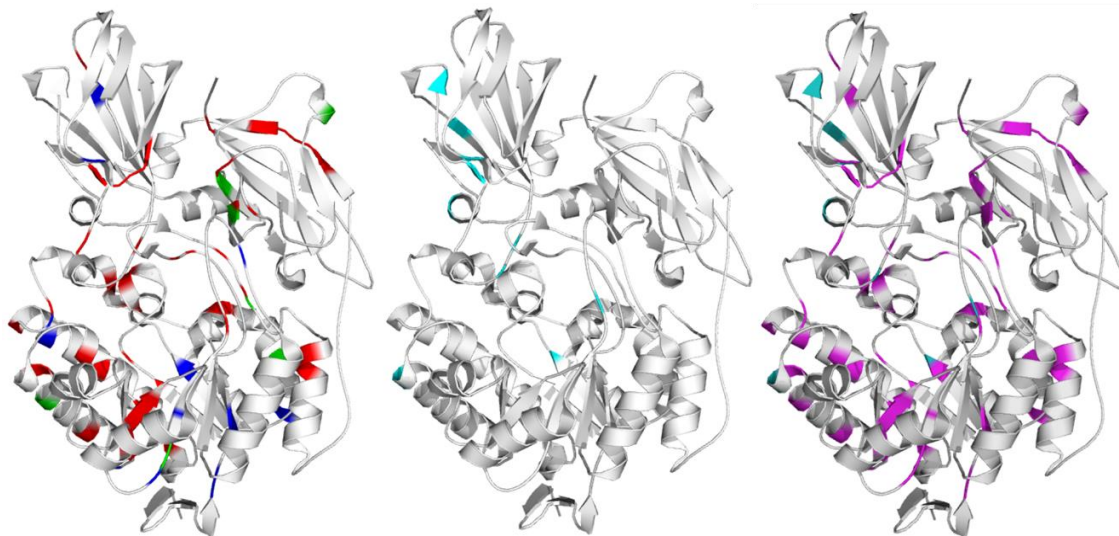


Figure 4. GLB1 with G_{M1}-gangliosidosis and Morquio B Disease mutations. Left: Monomer of GLB1 with location of G_{M1}-gangliosidosis mutations. Over 100 mutations have been discovered and G_{M1}-gangliosidosis can result from any of these point mutations. Disease is further categorized by severity and age of onset, Type I being the most severe form of the disease. Type I is in red, Type II is in green, and Type III is in blue. Center: Monomer of GLB1 with Morquio B mutations in cyan. Right: Monomer of GLB1 with both G_{M1}-ganglioside (magenta) and Morquio B (cyan) mutations. Figure generated using PDBID: 3THC.

and is classified into three categories, Type I, Type II, and Type III. Type I, or Infantile G_{M1} gangliosidosis, manifests within the first six months of life and is the most severe form of the disease. It is characterized by massive nervous system involvement, coarse facial features, skeletal dysplasia, visceromegaly, and rapid progression to death, usually within the first two years of life. Type II, or Juvenile G_{M1} -gangliosidosis, has a later onset, usually manifesting by age three, and has a slower disease progression. Type III, or Adult G_{M1} -gangliosidosis, has the slowest disease progression and manifests between ages three to thirty. Morquio B disease, or Mucopolysaccharidosis IVB, is characterized by massive skeletal changes, corneal clouding, and impaired cardiac function, and lacks any primary CNS involvement. There are no world-wide incidence data for Morquio B disease. Mutations for each disease are mapped onto monomer of GLB1 in Figure 4. Neither of these diseases currently have an effective treatment.

It is not clear why G_{M1} -gangliosidosis and Morquio B present such markedly different disease phenotypes but each result from mutations in the same enzyme. One cause for the difference may be in the delivery of substrates, G_{M1} -gangliosidosis and keratan sulfate (Figure 5). In order for cleavage of G_{M1} -gangliosidosis to occur, an activator protein is required, Saposin B (sap B) (Zschoche, et al. 1994, Paschke, et al.

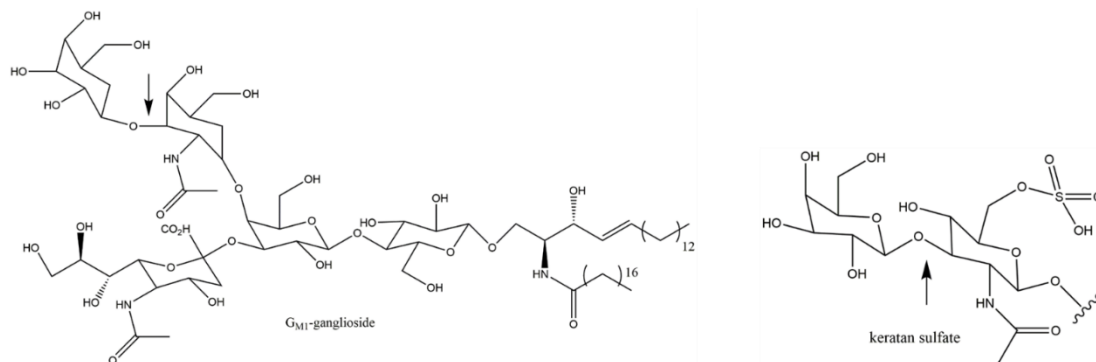


Figure 5. Substrates of GLB1. Left: G_{M1} -ganglioside, Right: keratan sulfate. Arrows indicate cleavage site by GLB1.

1982). Sap B is not required for the hydrolysis of keratan sulfate (Pshhezhetsky and Ashmarina, 2001). One study has shown that the Morquio B mutation W273L changes the affinity of GLB1 for keratan sulfate only (Oshima, et al. 1991), and there is no hindrance to G_{M1} -ganglioside metabolism. Future studies involving binding of natural substrates and sap B are needed to answer these questions.

Small Molecule Inhibitors of GLB1

The identification of potential small molecule inhibitors to be used as pharmacological chaperones for G_{M1} -gangliosidosis and Morquio B disease has been the topic of intensive research over the last decade, as no effective treatment is currently available for these diseases. Since the structure of GLB1 was not published until 2012, previous research was based on galactose, the product of the reaction, which was already known to be a competitive active site inhibitor (Figure 6A). Previous studies have shown that adding galactose to another glycosidase, α -galactosidase, can recover enough

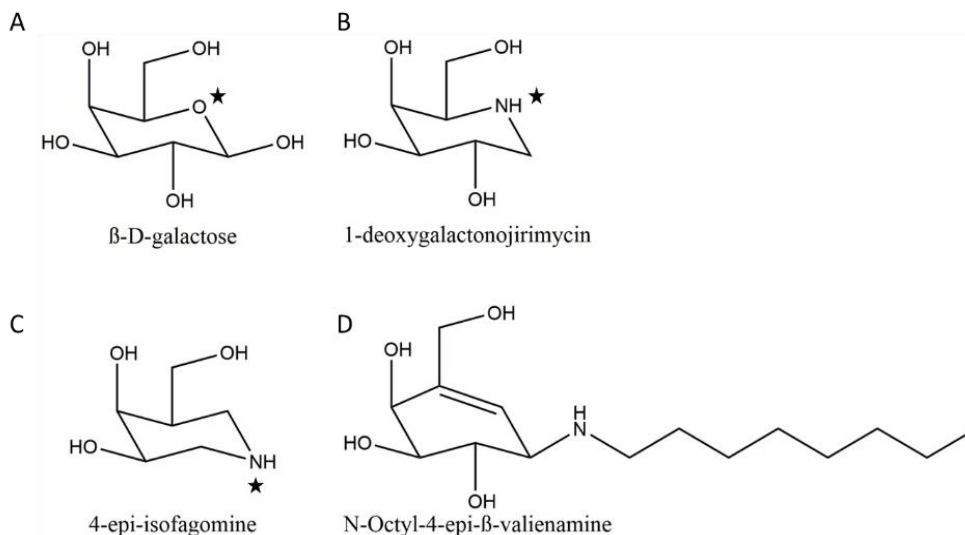


Figure 6. Structures of small molecule inhibitors of GLB1. A. The catalytic product, galactose, with stars showing relevance in comparison with the other molecules. B. DGJ, an iminosugar with neither α nor β specificity, but has an amine substituted for the heterocyclic oxygen. C. 4-epi-isofagomine is another iminosugar, with the amine in position 1. D. NOEV is a DGJ analog with additional hydrophobic characteristics added at position 1.

enzyme activity to degrade substrate in Fabry disease fibroblasts cells (Okumiya, et al. 1995). As a result of the success of galactose in Fabry diseased cells, galactose was tested for chaperoning activity in G_{M1} -gangliosidosis fibroblasts. In fibroblasts from infantile and juvenile G_{M1} -gangliosidosis patients, there was no inherent effect of the addition of galactose on the recovery of enzyme activity. However, in the adult G_{M1} -gangliosidosis, fibroblasts enzyme activity increased two to five times basal values at a concentration of 200 mM galactose (Caciotti, et al. 2009). In addition, COS-1 cells, (African green monkey kidney cells) that expressed the adult G_{M1} -gangliosidosis mutation R442Q also showed improved enzymatic activity, increasing 6.9-12% from control values after treatment with 200 mM galactose (Caciotti, et al. 2009).

In addition to galactose, another compound shown to be a tight binding inhibitor of α -galactosidase is 1-deoxygalactonojirimycin (DGJ) (Figure 6B). Although DGJ has been tested as an inhibitor of GLB1, its affinity is reduced compared to its affinity for α -galactosidase (Guce, et al. 2011, Clark, et al 2012). Furthermore, other galactose derivatives have been created and tested as possible small molecule inhibitors. Many of these compounds show micromolar IC_{50} values when tested with GLB1 (Fantur, et al. 2010, Froehlich, et al. 2011). The compound N-octyl-4-epi- β -valienamine (NOEV) (Figure 6D) has been the focus of several studies because this compound is a tight binding inhibitor of GLB1 and increases enzyme activity (Matsuda, et al. 2003). Another study also demonstrated the effectiveness of NOEV, finding 22 of 94 missense mutations in G_{M1} -gangliosidosis were responsive to NOEV chaperone treatment (Higaki, et al. 2011). A small library of isofagomine derivatives were tested as competitive inhibitors of various glycosidases (Kato, et al. 2011). In this study, the small molecule 4-epi

isofagomine (Figure 6C) was a good inhibitor of β -galactosidase from bovine liver and rat intestines, with IC_{50} values of 21 μ M and 0.51 μ M respectively (Kato, et al. 2011). Another study performed inhibition assays and crystallography with galactose, DGJ, NOEV (PBID: 3WEZ), and three new compounds, 5*N*, 6*S*-(*N*'-butyliminomethylidene)-6-thio-1-deoxygalactonojirimycin (6*S*-NBI-DGJ, PBID: 3WF0), 5*N*, 6*S*-(*N*'-butyliminomethylidene)-6-thiogalactonojirimycin (6*S*-NBI-GJ, PBID: 3WF1), and *N*-(*N*'-butylthiocarbamoyl)-1-deoxygalactonojirimycin (NBT-DGJ, PBID: 3WF2). Of these compounds NOEV had the highest inhibition with a K_i of 1.1 μ M (Suzuki, et al 2014).

Pichia pastoris

Pichia pastoris is a methylotropic yeast, capable of using methanol as its sole source of carbon through the induction of alcohol oxidase (AOX). In the presence of other carbon sources, the AOX proteins are virtually absent, but under induction conditions accounts for greater than 30% of cellular protein. *Pichia* contains two alcohol oxidase genes, *AOX1* and *AOX2*. Most of the alcohol oxidase activity results from expression from the *AOX1* gene with the phenotype methanol utilization plus (Mut^+), but if disrupted, *Pichia* are still capable of utilizing methanol as its sole carbon source via the *AOX2* gene. However, it is at a slower rate leading to the phenotype methanol utilization slow (Mut^S). *Pichia* has also been largely sought after as a protein expression system due to its ability to generate ultra-high cell density during fermentation (>130 g dry cell weight/L) (Sreekrishna and Kropp 1996). Two *Pichia* strains were used in these studies, X-33 and GS115. X-33 is a wild-type *Pichia* strain with the ability to grow in complex and minimal media. The GS115 strain contains a mutation in the histidinol dehydrogenase gene (*his4*) that prevents histidine synthesis. This strain has the ability to

grow on both complex media and minimal media supplemented with histidine. Both of these strains have an intact *AOX1* gene, and thus should produce recombinant yeast with the phenotype Mut⁺.

Research Aims

The aim of my research is to express, purify and evaluate the enzymatic parameters of human lysosomal β -galactosidase expressed from *Pichia pastoris*. The rationale behind creating this system is to 1.) Improve upon the expression observed in insect cells and 2.) Create a protein sample that can be easily deglycosylated and processed to the mature form for crystallography experiments. Crystallography of GLB1 previously purified from insect cells was unsuccessful; with this construct we are much closer to obtaining crystals. The published crystal structure was from recombinant protein expressed in *Pichia*, therefore this should be a valid expression system (Ohto, et al. 2012). From this expression system recombinant GLB1 can then be used in not only crystallography experiments, but other biochemical and structural studies.

CHAPTER 2

MATERIALS AND METHODS

Molecular Biology

The *GLB1* gene with a C-terminal four glycine spacer and hexahistidine tag had previously been inserted into the pIB/V5 vector for insect cell expression (Rivera-Colon, 2013). To insert into pPICZ α A vector (Invitrogen) for *Pichia* expression, PCR was performed. This PCR created a fragment containing the *GLB1* gene (residues 24-677) and attached spacer and tag, inserted downstream of the *Saccharomyces cerevisiae* α -mating factor signal sequence (Figure 7). The construct also included XhoI and SacII restriction enzyme sites for cloning purposes. PCR primers used were as follows: forward 5'ACTATCTCGAGAAAAGAGAGGCTTTGCGCAATGCCACCA 3'; reverse 5' ACGTTCCGCGGTTTAATGGTGATGGTGATGGTGACCTCCACCTCCTACA 3'. PCR was performed using Phusion polymerase (New England Biolabs) with final concentrations of 1X GC Buffer (NEB), 250 μ M dNTPs (NEB), 625 μ M forward and

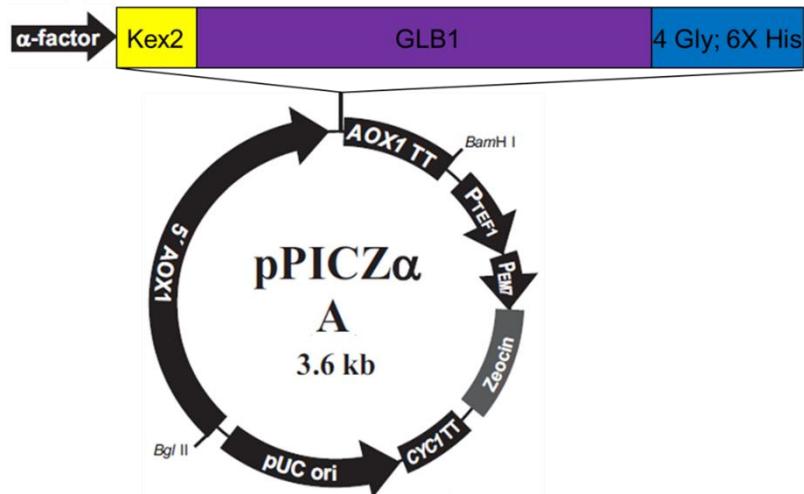


Figure 7. GLB1 construct inserted into the pPICZ α A vector. The GLB1 gene was inserted into the pPICZ α A vector using restriction enzymes. This construct contained a C-terminal four glycine spacer followed by a hexahistidine tag for affinity purification. A Kex2 signal sequence was also included for protein secretion.

reverse primers, 0.5 ng template DNA with a 20 μ L total reaction volume. Successful PCR product was confirmed via agarose gel electrophoresis using 0.8% agarose. Following PCR, products were purified using a Wizard SV gel and PCR clean up kit (Promega). PCR products and the pPICZ α A vector (Invitrogen) were then digested with XhoI and SacII (NEB) overnight at 37°C, and complete digestion confirmed via agarose gel electrophoresis. After gel purification, the empty vector was dephosphorylated using Antarctic phosphatase (NEB) to minimize background transformants. The reaction proceeded at 37°C for one hour followed by heat inactivation at 65°C for 20 minutes. Insert and vector were ligated at a 3:1 ratio using a total of 100 ng DNA. Quick Ligase Kit (NEB) was used and the reaction incubated at room temperature for five minutes. Ligation products of insert and vector and dephosphorylated empty vector were transformed into TOP10 *E. coli* cells (Invitrogen) and plated on low-salt LB agar plates containing 25 μ g/mL Zeocin. These were incubated at 37°C overnight, and then checked for bacterial growth. Several colonies were selected and subjected to colony PCR where insertion of the *GLB1* gene was confirmed. Four colonies from this PCR were then selected and sent out for sequencing to confirm the correct insertion of *GLB1* into the pPICZ α A vector.

Yeast Transformation

Two host strains of *Pichia pastoris*, X-33 and GS115, were selected for transformation. The X-33 genotype is wild type resulting in a Mut⁺ *Pichia* phenotype, whereas the GS115 genotype is *his4* resulting in a His⁻, Mut⁺ *Pichia* phenotype. The lithium chloride method for yeast transformation was used with 8 μ g of plasmid per

transformation. Cells were plated two and seven hours after transformation onto YPDS plates containing 100 µg/mL Zeocin.

To confirm insertion of *GLB1/pPICZαA* vector, colony PCR of yeast transformants was performed. PCR was performed on select colonies with the following conditions: 1X Taq Polymerase reaction buffer (NEB), 2.5 mM MgCl₂, 2.5 mM dNTPs (NEB), 0.5 mM forward and reverse primers, and Taq polymerase (0.16 U/µL) with ddH₂O added to a final reaction volume of 50 µL. Select colonies were also screened for Mut phenotype, to see where the vector had inserted in the yeast genome, and how the transformants grew under induction conditions. Colonies were typically Mut⁺, but others studies have shown a small number of colonies to be Mut^S (Sreekrishna and Kropp 1996). These colonies were first patched onto minimal media plates (with and without histidine) containing methanol, followed by patching on minimal media plates (with and without histidine) containing dextrose.

Protein Expression

To assess for GLB1 expression, four colonies were selected for small-scale expression at both pH, 4.5 and 6.0. These pHs were selected, as the recommended pH 6.0 (Invitrogen Easy Select manual) and pH 4.5, the pH of the lysosome, where the protein is expected to be most stable. A single colony was used to inoculate 25 mL buffered complex media containing glycerol (BMGY) containing 1X penicillin and streptomycin antibiotics shaking at 250 rpm at 30°C and grown to an OD₆₀₀ of ~2-6 (approximately 20 hours). These cells were then harvested at 3000 x g for five minutes. Protein expression was induced by resuspending cells in buffered complex media

containing methanol (BMMY) to an OD₆₀₀ of 1.0 (approximately 100-200 mL). Cultures grew for 96 hours at 27°C shaking at 250 rpm. 100% methanol was added every 24 hours to a final concentration of 0.5% for induction to be maintained. 1 mL time points were taken at 6, 12, 24, 48, 72, and 96 hours and assayed for GLB1 expression. Larger scale culturing was performed as described in the Invitrogen Easy Select manual.

X-gal Activity Assay

GLB1 expression assays were performed using a dot-blot activity assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the substrate. X-gal is cleaved by GLB1 (Figure 8) yielding galactose and 5-bromo-4-chloro-3-hydroxyindole, which then dimerizes and oxidizes to 5, 5-dibromo-4, 4'-dichloro-indigo, displaying a bright blue color. 50 μL of supernatant was blotted onto nitrocellulose, gravity filtered through the membrane and then incubated in 10 mM acetate pH 4.5, 120 mM sodium chloride containing 50 μL of 100 mg/mL X-gal at 37°C overnight. The appearance of blue color indicated the presence of GLB1 in the sample.

Protein Purification

After 96 hours cells were pelleted via centrifugation at 4000 rcf for 5 minutes. The supernatant was then decanted and re-centrifuged twice at 5000 rcf for 15 and 45 minutes to remove any remaining cell debris. The supernatant was concentrated and buffer exchanged via tangential flow through 10 kDa filter. After concentrating ten-fold, the sample was buffer exchanged into wash buffer for nickel-affinity chromatography (50 mM phosphate pH 7.0, 250 mM sodium chloride). The sample was loaded onto a 5 mL nickel fast flowcolumn (GE), to which the hexahistidine tag on GLB1 binds. GLB1 was

eluted with a gradient of up to 400 mM imidazole, which competes with the His-tagged GLB1 for binding on the nickel column. 1.25 mL fractions were collected and analyzed for GLB1 presence and activity. The fractions containing GLB1 were pooled for assay or further purification. Analyses of purifications were performed using 10% SDS-PAGE gels.

Deglycosylation

Deglycosylation was performed to remove the glycosylations from GLB1, as *Pichia* has a tendency to hyperglycosylate secreted proteins. EndoH (NEB) was chosen as the endoglycosidase as results have shown it to completely deglycosylate GLB1 (Usui, et al 2011). Purified protein was buffer exchanged into 20 mM sodium acetate pH 5.1, 100 mM sodium chloride before 500 U of EndoH was added. This reaction was incubated at room temperature overnight. After deglycosylation GLB1 was subjected to affinity purification using a *p*-aminophenyl β -D thiogalactopyranoside agarose column (Figure 9). Sample was buffer exchanged into equilibration buffer (20 mM sodium acetate pH 5.1, 100 mM sodium chloride) and then incubated with 1 mL of packed matrix for ~30 minutes before washing with 50 column volumes of equilibration buffer. GLB1 was then eluted using 20 mM sodium acetate pH 5.1, 100 mM sodium chloride, 1 M

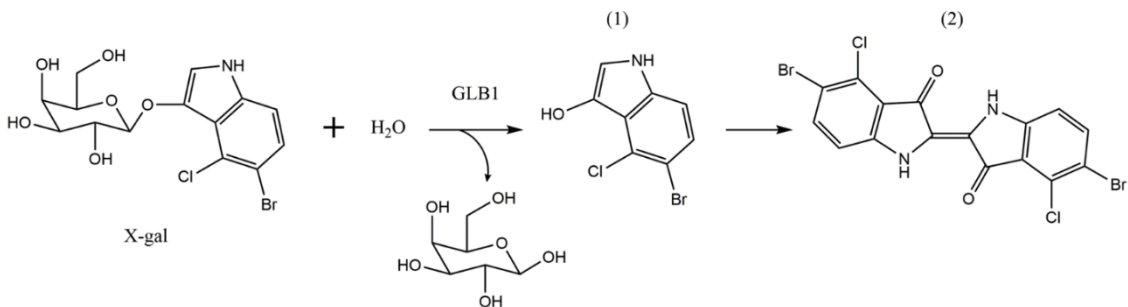


Figure 8. X-gal reaction mechanism. GLB1 hydrolyzes X-gal, producing galactose and 5-bromo-4-chloro-3-hydroxyindole (1). This then dimerizes and is oxidized to form an insoluble, blue precipitate (2).

galactose. Fractions containing GLB1 were pooled and concentrated for use in assays.

Analysis of purification was performed using 10% SDS-PAGE.

Trypsin Proteolytic Cleavage

In order to achieve the mature form of GLB1, limited trypsin proteolysis was performed. The published procedures in Usui et al. 2011 were followed, and GLB1 was buffer exchanged into 50 mM phosphate buffer pH 5.1, 120 mM sodium chloride. Limited proteolysis was initiated by the addition of 1:25 (w:w) trypsin. The reaction proceeded at 37°C for 30 minutes before benzamidine was added to a final concentration of 1 mM to stop the reaction. Analysis of limited proteolysis was performed using 10% SDS-PAGE.

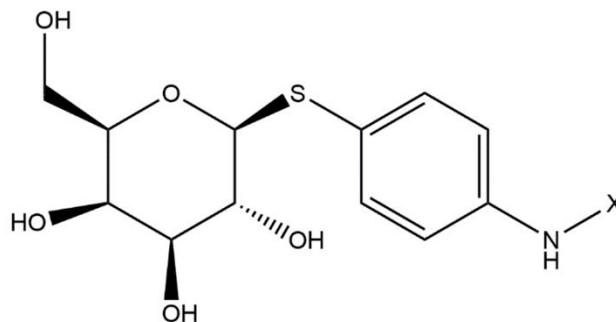


Figure 9. p-aminophenyl β -D thiogalactopyranoside agarose matrix. Non-hydrolyzable affinity matrix used for GLB1 affinity purification.

Enzyme Kinetics

Enzyme kinetics were performed using a synthetic substrate, *para*-nitrophenyl- β -D-galactopyranoside (*p*NP- β -gal). GLB1 cleaves this substrate into D-galactose and *para*-nitrophenol (*p*NP). When placed under the basic conditions of the stop buffer, *p*NP deprotonates to the *para*-nitrophenolate ion. This reaction shown in Figure 10 produces a yellow color which absorbs light at 400 nm. Therefore, absorbance was read at 400 nm, then converted to *p*NP concentration to determine activity in all assays. Twelve substrate concentrations were prepared by combining *p*NP- β -gal from 0.1 mM - 6 mM, 0.2 M phosphate-citrate buffer pH 4.0, 0.1% bovine serum albumin (added as a crowding agent), and 0.133 μ g/mL GLB1. The reaction proceeded at 37°C for 25 minutes, with time points taken at 0, 5, 10, 15, 20, and 25 minutes. They were then diluted 30 fold in 200 mM borate buffer, pH 9.8. Product concentrations were determined by converting the absorbance as described above. From these concentrations kinetic parameters were

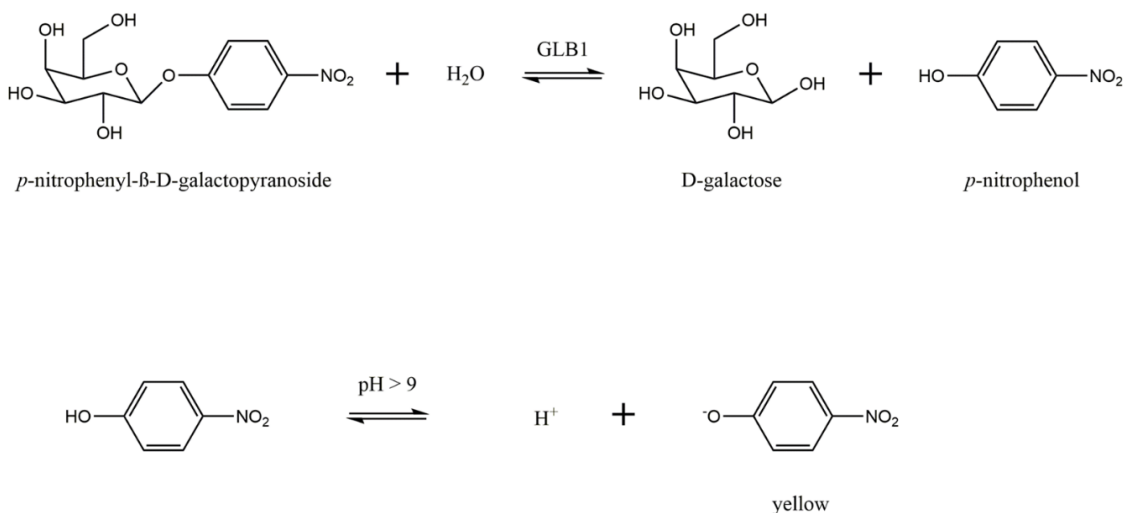


Figure 10. Reaction between the synthetic substrate *para*-nitrophenyl- β -D-galactopyranoside and GLB1. When GLB1 reacts with *p*NP- β -gal, it cleaves off the *p*-nitrophenol. When placed in a basic environment this ion then produces a yellow color which absorbs light at 400 nm.

determined using Kaleidagraph graphing software. K_M and V_{max} were calculated using the following Michaelis-Menten formula:

$$V_0 = \frac{V_{max} \times [S]}{K_M + [S]}$$

For inhibition assays, eight inhibitor concentrations were prepared by combining the inhibitor (galactose, DGJ, or 4-epi isofagomine) at various concentrations, 0.2 M phosphate-citrate buffer pH 4.0, 0.2 mM *p*NP- β -gal substrate, 0.1% bovine serum albumin (added as a crowding agent), and 0.266 μ g/mL GLB1. The reaction proceeded at 37°C for 50 minutes, with time points taken at 0, 10, 20, 30, 40, and 50 minutes. They were then diluted 30 fold, in 200 mM borate buffer, pH 9.8. Product concentrations were determined by converting the absorbance as described above. From these concentrations, IC_{50} values were determined using sigmoidal curve fit in Kaleidagraph graphing software, giving concentrations at which half of the enzyme is inhibited. Inhibition constants (K_i) were then calculated using the following equation (Copeland, 2000).

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_M} \right)$$

The inhibition constants measure potency and indicate if the tested small molecules reach the threshold to be considered for pharmacological chaperone therapy.

CHAPTER 3

RESULTS

Insect cell inhibition assays

Previously in the lab insect cell purified GLB1 had been assayed against three small molecule inhibitors, galactose, DGJ, and 4-epi isofagomine to test the inhibitory effect of these small molecules (Rivera-Colón, 2013). To confirm these data, the inhibition assays were repeated at pH 4.5 using insect cell purified GLB1. We see from Figure 11 that galactose is a weak binding inhibitor of GLB1 with a K_i of 1.8 mM, and DGJ and 4-epi isofagomine are strong inhibitors with K_i s of 4.6 μ M and 4.1 μ M, respectively.

From these data we see that while 4-epi isofagomine was previously determined to be a 5-fold tighter inhibitor than DGJ; their inhibitory effects are comparable in this study. It may be that while they show similar inhibitory activity, 4-epi isofagomine will create a stronger interaction, as hypothesized through our structural modelling, and thus greater stability within the protein. 4-epi isofagomine also looks like it will create more favorable interactions in the active site, particularly with the catalytic nucleophile. In α -

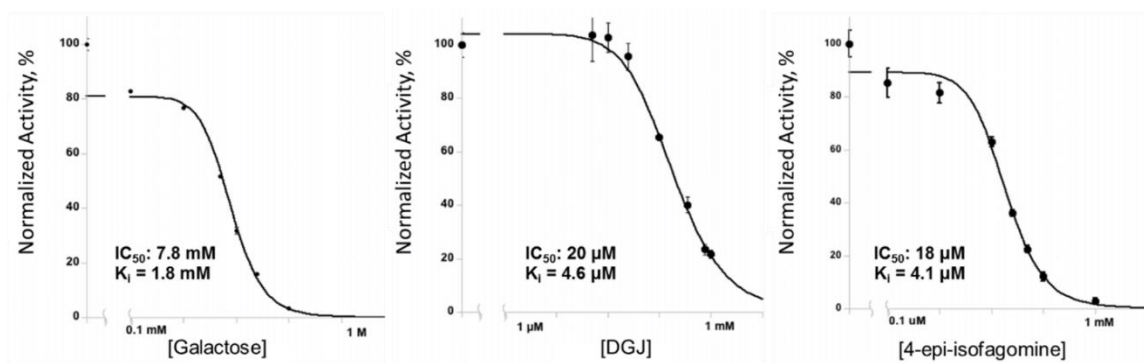


Figure 11. Inhibition of GLB1 activity by pharmacological candidates. Activity was normalized against enzyme activity in the same conditions without inhibitor. From left to right inhibition curves are shown for galactose, DGJ, and 4-epi-isofagomine. The IC₅₀ values for these compounds are 7.8 mM, 20 μ M, and 18 μ M respectively. The K_i values for the compounds are 1.8 mM, 4.6 μ M, and 4.1 μ M respectively.

galactosidase the tight binding seen is caused mainly via the interaction between D170 and the nitrogen in the heterocyclic ring of DGJ. However, in GLB1, the catalytic nucleophile E268 is predicted to create a tighter binding interaction when the heterocyclic nitrogen is moved to the one position, as seen in 4-epi-isofagomine. Atomic resolution of this interaction will give us definitive answers as to the interactions occurring in the active site of GLB1 bound with 4-epi-isofagomine.

Gene insertion and yeast transformation

The GLB1 gene containing a four glycine spacer, hexahistadine tag, and Kex2 signal sequence were successfully inserted into the pPICZ α A vector. Results were confirmed via colony PCR and sequenced (Figure 12). Successful yeast transformation occurred into two *Pichia* strains, X-33 and GS115, confirmed again by colony PCR (Figure 12). All tested transformants were also confirmed to express the Mut⁺ phenotype.

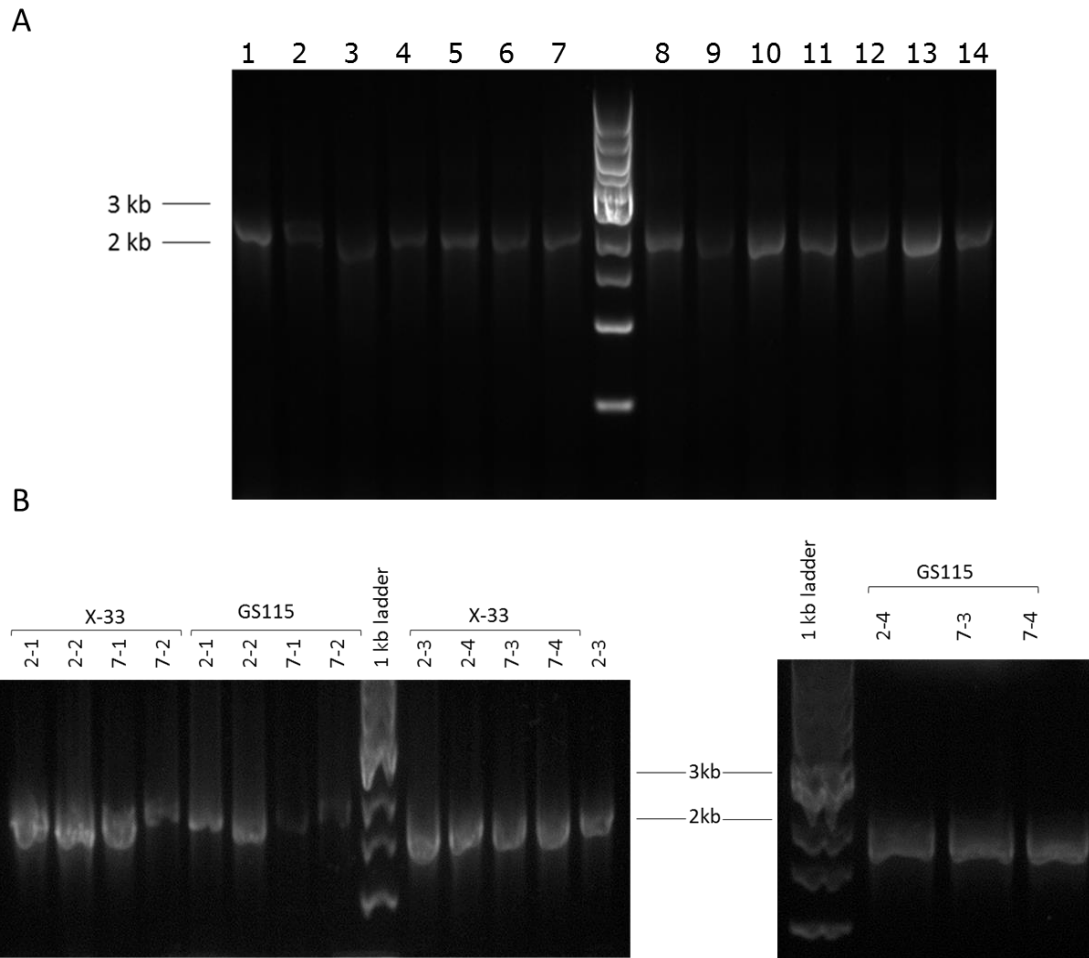


Figure 12. Molecular biology of GLB1 insertion into pPICZ α A vector. A. Colony PCR of Zeocin resistant *E. coli* transformants. Colonies 8 and 10 were found to have the correct sequence. B. Colony PCR of Zeocin resistant *Pichia* transformants. Colonies X-33 2-1, 2-2 and GS115 7-3, 7-4 were then tested for GLB1 expression.

Protein expression and purification

Following the 96 hour expression time course, 50 μ L samples of supernatant were blotted onto nitrocellulose to be assessed for GLB1 expression via X-gal activity assay. Results are shown in Figure 13 .

As shown, all strains from the X-33 parent strain began expressing GLB1 at around the 12 hour mark, and continued until assay saturation around 48-72 hours. Strains from the GS115 parent strain had not expressed GLB1 until around the 48 hour time point, reaching assay saturation around 72-96 hours.

After induction tests, the supernatant containing protein was harvested then buffer exchanged and concentrated via tangential flow. The clarified supernatant was run on a 5 mL nickel column, and the resulting chromatogram is shown in Figure 15. Fractions

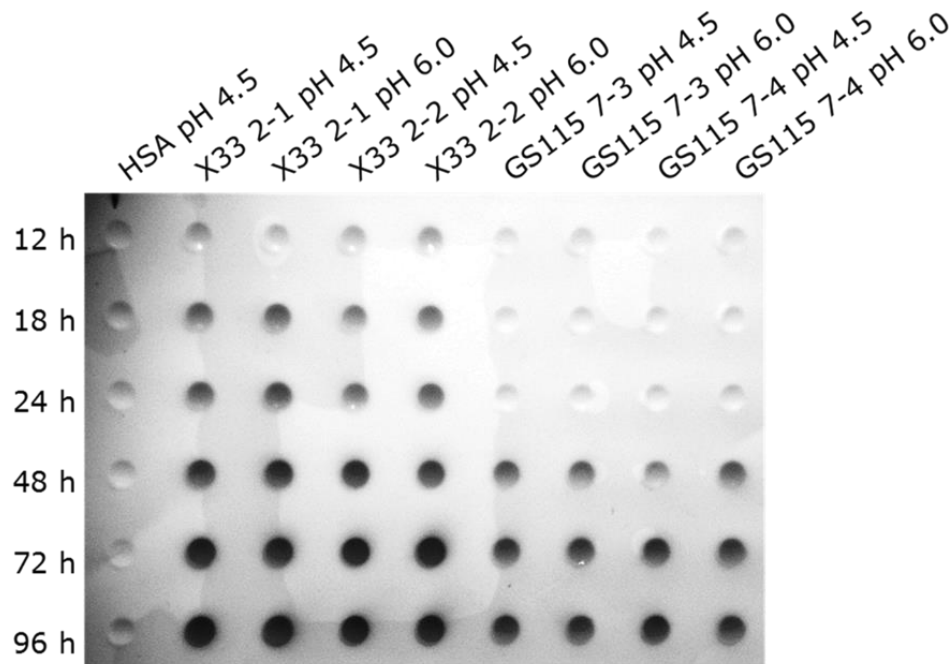


Figure 13. Time course study of Pichia expressed recombinant GLB1. At the specified time points 1 mL of culture was spun down, and the supernatant analyzed for GLB1 expression. 50 μ L of supernatant was blotted on nitrocellulose, then incubated with x-gal to determine GLB1 expression. Human serum albumin (HSA) was expressed as a control.

containing GLB1 were pooled and concentrated, the level of purity shown in Figure 15. These fractions were then deglycosylated using EndoH. Results of deglycosylation are shown in Figure 14. Following deglycosylation, GLB1 was further purified using a *p*-aminophenyl β -D thiogalactopyranoside agarose column. Each purification produced about 0.05 mg/mL of purified protein per 100-200 mL of supernatant, an improvement over insect cell expressed recombinant GLB1.

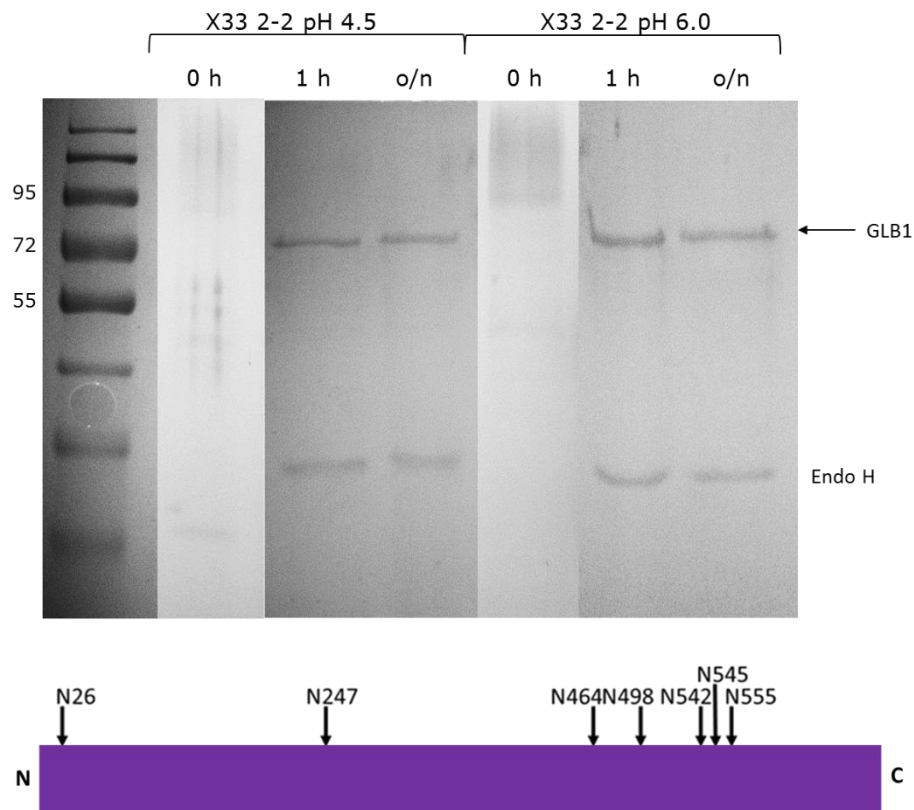


Figure 14. Deglycosylation of GLB1 using EndoH. GLB1 was deglycosylated using EndoH. The glycosidase activity was monitored with time points taken at one hour and then overnight. Below is a cartoon depicting glycosylation sites on GLB1.

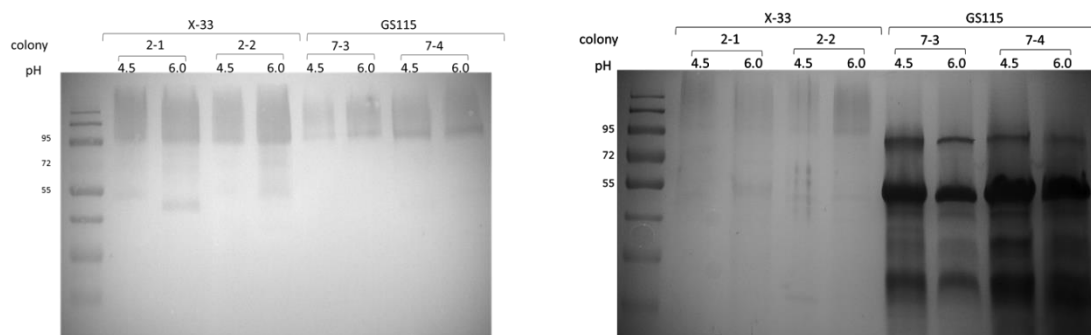
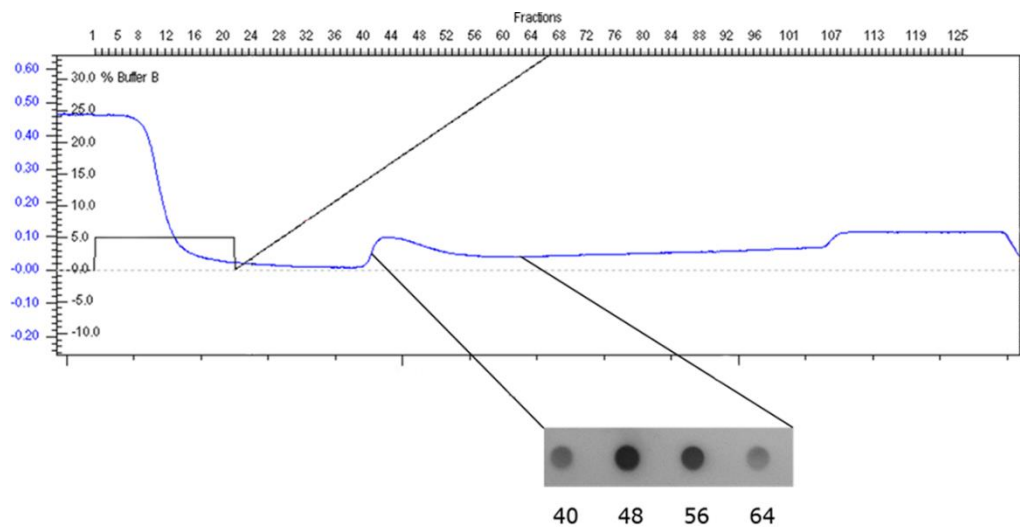


Figure 15. Purification of *Pichia* expressed recombinant GLB1. Top: chromatogram of nickel affinity purification. Peak corresponds to GLB1 expression as confirmed via X-gal assay. Bottom left: Western blot of concentrated, purified GLB1 samples. After purification, peaks containing GLB1 were pooled and concentrated. These concentrated samples were then separated by SDS-PAGE, transferred, and blotted with anti-GLB1 antibody. As shown, all samples contain GLB1. Bottom right: concentrated, purified GLB1 samples. Samples that were blotted were also subject to SDS-PAGE followed by GelCodeBlue staining to assess protein purity. Samples from the X-33 parent strain are highly glycosylated, and relatively pure. Samples from the GS115 strain are less hyperglycosylated, but still very impure.

Generation of Mature GLB1

The mature form of GLB1 was generated via limited proteolysis with trypsin. After 30 minutes the reaction was quenched using benzamidine. When run on a reducing SDS-PAGE, mature GLB1 runs in two fragments at approximately 64 and 20 kDa as seen in Figure 16.

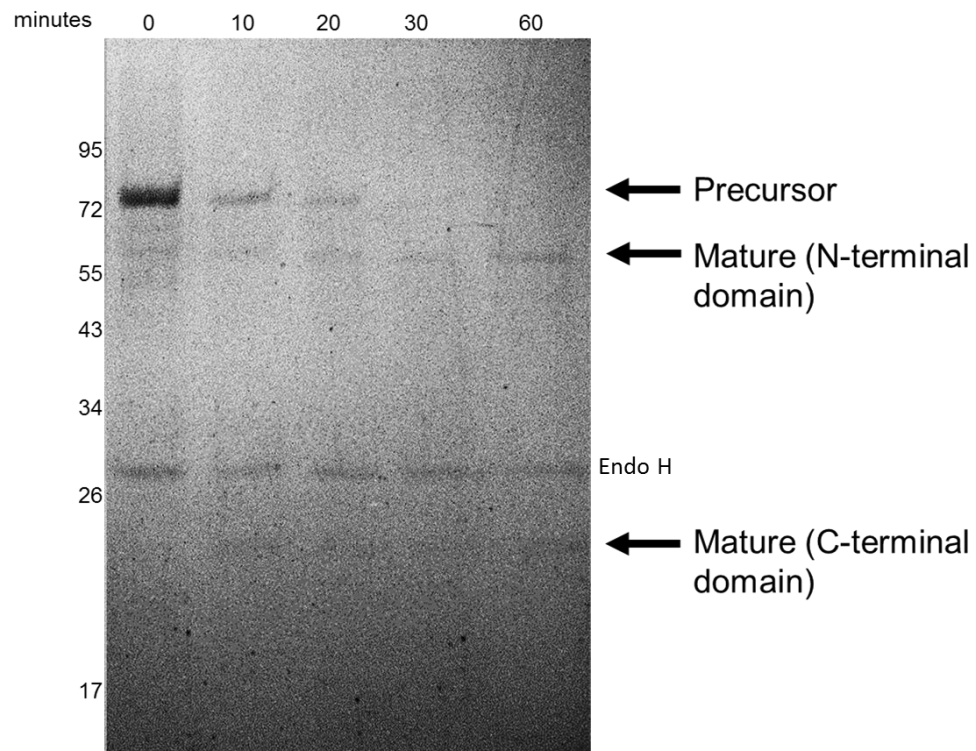


Figure 16. Limited proteolysis of GLB1. After 30 minutes incubation with trypsin, we see the N-terminal domain mature form of GLB1 appearing at approximately 64 kDa, and the C-terminal domain appearing at approximately 20 kDa. Labels to the left are in kDa.

Enzymatic activity of *Pichia* produced GLB1

Results of the Michaelis-Menten kinetic assay performed with *p*NP- β -gal are shown in Figure 17. I determined the K_M to be 0.82 ± 0.2 mM, V_{max} 0.002 ± 0.0002 mM/min, and k_{cat} 18.3 ± 1.83 /sec. This is comparable with other reported enzymatic parameters against synthetic substrate 4-methylumbelliferyl- β -D-galactoside (4MU- β -gal), and 2, 4-dinitrophenyl- β -D-galactoside as seen in Table 1 (Zhang, et al. 1994).

Table 1. Enzymatic characterization of recombinant GLB1

Recombinant GLB1	Substrate	K_M	V_{max}	k_{cat}
Chinese Hamster Ovary	4MU- β -gal	0.29mM	989 mmol/h/mg	-
Chinese Hamster Ovary	2,4-dinitrophenyl- β -D-galactose	0.73 mM	888 mmol/hr/mg	-
<i>Pichia pastoris</i>	<i>p</i> NP- β -gal	0.82 ± 0.2 mM	0.002 ± 0.0002 mM/min	18.3 ± 1.83 /sec

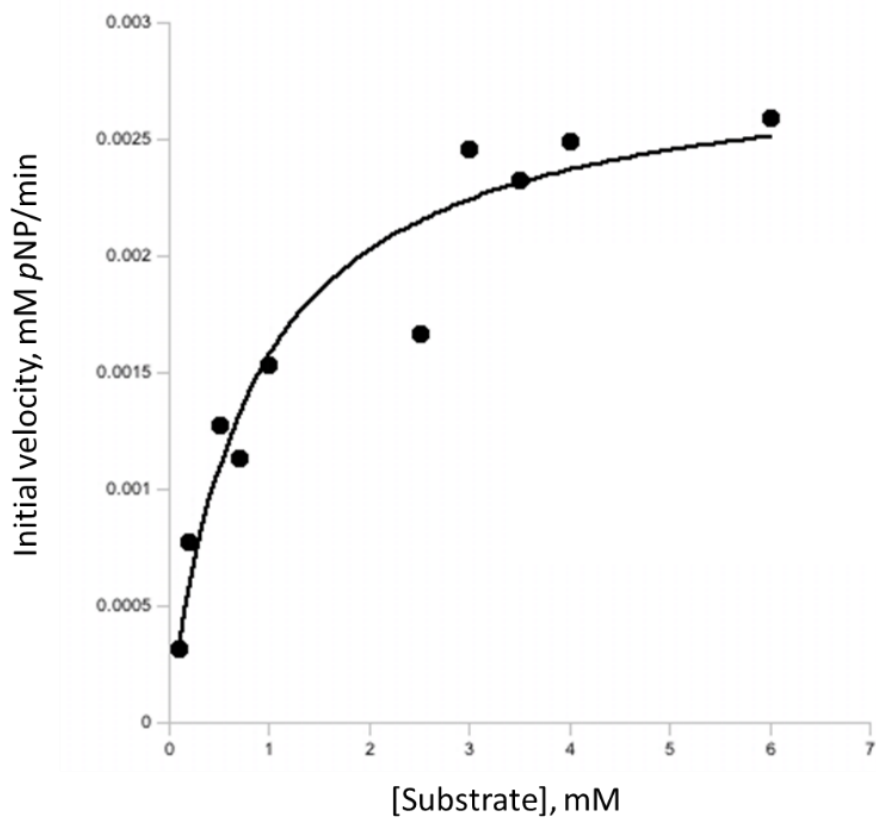


Figure 17. Michaelis-Menten plot of GLB1 activity. The enzymatic parameters of GLB1 are as follows: K_M to be 0.82 ± 0.2 mM and V_{max} 0.002 ± 0.0002 mM min^{-1} .

CHAPTER 4

CONCLUSIONS

I report that galactose, DGJ, and 4-epi isofagomine are inhibitors of GLB1 with K_i values of 1.8 mM, 4.6 μ M, and 4.1 μ M respectively. From these data, I conclude that DGJ and 4-epi isofagomine are micromolar inhibitors of GLB1, which might make them candidates for pharmacological chaperone therapy. If we compare these results to those published in Suzuki et al 2014, the K_i for the small molecule DGJ of 4.6 μ M is lower than the 61.8 μ M they report. Our value meets the 10 μ M threshold outline as required in order for potential pharmacological chaperones to proceed to cellular assays (Fan, 2008). The almost twelve-fold difference in inhibition may be due to the different forms of GLB1 used. In our experiments, the precursor form of GLB1 was used, in comparison to the processed mature GLB1. If this difference in inhibition constants between precursor and mature GLB1 holds true, it has positive implications for pharmacological chaperone therapy. The optimum pharmacological chaperone has a high affinity for protein outside the lysosome, yet lower affinity for the protein once it reaches the lysosome. This is usually achieved through the change in pH from the ER and trafficking compartments to the lysosome, but in the case of GLB1, the processing from precursor to mature form could also be utilized.

We have also shown that GLB1 can be expressed, purified, and processed from *Pichia pastoris*. *Pichia* expression has many advantages over insect cell expression. Previously in the lab, insect cells had produced 0.1 mg of purified protein per liter of culture. While just meeting the threshold for expression, this small amount of protein per liter was difficult to replicate and would require a very large culture to produce enough

protein suitable for crystallography experiments. In *Pichia* we have observed approximately 0.05 mg of purified protein per 100 milliliters of cell culture, a five-fold improvement over insect cell expressed GLB1. This expression system needs further optimization, as the amount of expression does not scale linearly with the current protocol. A logical next step would be to try fermentation, as there are many reports of high protein yield from fermented *Pichia*. It may also be worthwhile to transform GLB1 into the KM71H strain of *Pichia*, creating a Mut^S strain. It may be that this phenotype will produce even higher levels of recombinant GLB1. *Pichia* also allows for GLB1 to be easily deglycosylated by commercially available glycosidases. Due to the nature of insect cell glycosylation machinery, the glycoproteins are fucosylated, and thus resistant to enzymatic removal (Dojima, et al. 2009, Gouveia, et al. 2009). The removal of glycosylations is optimal, as one of the future directions for this project is crystallization of GLB1, which requires a highly purified uniform protein sample. Furthermore, the recombinant *Pichia* GLB1 can also be easily processed from precursor to mature form. Previous attempts in the lab have only produced partial proteolysis of GLB1, whereas the use of trypsin in *Pichia* expressed-GLB1 yields a uniform fully mature form of the protein. It is also the experience of the lab that the precursor form of GLB1 will not yield crystals, thus this construct, which is easily manipulated from precursor to mature form, is ideal for future crystallography experiments. Overall, the expression of GLB1 from *Pichia* is successful and worth pursuing for future experiments.

Future studies for this project include crystallization of mature GLB1 in complex with the small molecule 4-epi-isofagomine. This structure will help provide insight into the molecular interactions occurring on the atomic level, allowing us to utilize rational

drug design to find a better candidate for pharmacological chaperone therapy in G_{M1} -gangliosidosis and Morquio B disease. In addition to structural studies, cellular assays with both wild-type and mutant protein showing stabilization of GLB1 with small molecules are needed to confirm *in vitro* effects *in vivo*. Other studies that may be carried out with the recombinant GLB1 involve probing questions surrounding the multi-enzyme complex. The literature is convoluted as to the actual size of this complex and whether it contains not only PPCA, NEU1, and GLB1, but possibly another lysosomal enzyme, N-acetylgalactosamine-6-sulfate sulfatase (GALNS) (Bonten, et al. 2014). It may also be interesting to study the effects of GLB1 disease mutations on complex formation, whether or not this may attribute to the unique genotypes of G_{M1} -gangliosidosis and Morquio B disease. Mechanistic studies in terms of how disease genotypes may be linked to substrate delivery may also be of interest. G_{M1} -ganglioside is delivered via saposin B, whereas keratan sulfate requires no chaperone for GLB1 metabolism. If these mechanisms can be uncovered, the molecular mechanism for mutations in one enzyme causing two diseases may be brought to light.

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