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Development of a Multiplex Exoglycosidase Assay for Diagnosis of Oligosaccharidoses using Tandem Mass Spectrometry

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Development of a Multiplex Exoglycosidase Assay for Diagnosis of Oligosaccharidoses using Tandem Mass Spectrometry

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Abstract

Oligosaccharidoses are Lysosomal Storage Disorders (LSDs) that result from mutations in genes encoding exoglycosidases, leading to accumulation of unmetabolized N-linked oligosaccharides within lysosomes. Age at onset and rate of disease progression vary among patients. Diagnosis based solely on clinical presentation is often challenging because of overlapping clinical symptoms between these disorders. The aim of this research is to use tandem mass spectrometry (MS/MS) to establish a multiplex method to measure exoglycosidase activities, in dried blood spots (DBS), involved in the degradation of N-linked oligosaccharides using natural substrates. Current fluorometric assays for each exoglycosidase using specific 4-methylumbelliferyl (4MU) substrates allow enzyme activities to be determined separately from a variety of human tissue sample types. A universal buffer was established by comparing these assay conditions to allow multiplexing of the exoglycosidases in a single vial. Initial attempts to develop an enzyme activity assay using disaccharides as the starting substrate and by monitoring unique monosaccharide products by MS/MS after exposure to an enzyme source from cultured skin fibroblasts were unsuccessful due to interfering endogenous hexose isomers. Taking another approach, multiplexing was successfully demonstrated for β-Galactosidase and β-Hexosaminidase using alternative substrates. 4MU and paranitrophenol (PNP) conjugated to particular monosaccharides allowed 4MU and PNP products to be measured and enzyme activities to be calculated. Here, we provide a proof of principle that MS/MS technology can allow simultaneous multiplexing of several enzyme activities using distinctive starting substrates. A multiplex assay for the remaining exoglycosidases can still permit the development of an Oligosaccharidoses screening test to assist clinical diagnosis.

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List of Abbreviations

2AMAC	2-aminoacridone
4MU	4-methylumbelliferyl
AE	Active enzyme
ABEE	4-aminobenzoic acid ethyl ester
Asn	Asparagine
CE	Collision energy
CH_3CN	Acetonitrile
CHEO	Children's Hospital of Eastern Ontario
CH_2O_2	Formic acid
CID	Collision induced dissociation
CPRI	Child Parent Resource Institute
CV	Cone voltage
DAABD-MHz	4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole
DBS	Dried blood spots
DHZ	Dansylhydrazine
ER	Endoplasmic reticulum
ESI	Electrospray ionization
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
Glu	Glucose
HPLC	High performance liquid chromatography
Lys	Lysine
IE	Inactive enzyme
IEM	Inborn errors of metabolism
LSD	Lysosomal Storage Disorder
Man	Mannose
Man-6-P	Mannose-6-Phosphate

MeOH	Methanol
MRM	Multiple reaction monitoring scan mode
MS1	First mass analyzer
MS2	Second mass analyzer
MS/MS	Tandem mass spectrometry
MS mode	Precursor ion scan mode
MS/MS mode	Product ion scan mode
m/z	Mass to charge ratio
NH_4OAc	Ammonium acetate
NSO	Newborn Screening Ontario
P/D	Parent to daughter transition
Phe	Phenylalanine
PKU	Phenylketonuria
PMP	1-phenyl-3-methyl-5-pyrazolone
PNH	Phenylhydrazine
PNP	Paranitrophenol
Ser	Serine
Sia	Sialic acid
Thr	Threonine
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography

Introduction

1.1 Overview of Lysosomal Storage Disorders (LSDs)

1.1.1 Classification of Oligosaccharidoses

Oligosaccharidoses or Glycoproteinoses are a group of rare human diseases that are a subset of the Lysosomal Storage Disorders (LSDs), which belong to the broader collection of inborn errors of metabolism (IEM). LSDs consist of at least 50 heterogeneous inherited genetic diseases, with a collective prevalence estimated as high as 1 in 7000-8000 live births (Meikle et al. 1999, Pastores 2010). Most LSDs are inherited in an autosomal recessive manner and result from a mutation in a gene that encodes an intralysosomal acid hydrolase, leading to a disruption in the catabolism of specific macromolecules. However, other LSDs could result from a defect of proteins involved in lysosomal enzyme targeting or in the transport of macromolecules across the lysosomal membrane. These may include membrane proteins, transporter proteins, enzyme coactivators and proteins that process specific lysosomal enzymes for proper localization (Pastores 2010, Neufeld, Lim & Shapiro 1975). Under these circumstances, the resultant build-up of substrates in various cellular organelles will cause a loss of function in the cell and ultimately in one or several crucial organs in the body. Clinical features are very much dependent upon the rate and extent of accumulated undegraded substrates and tend to differ among patients with any given LSD (Pastores 2010).

LSDs are generally classified according to the nature of the stored material, but some are grouped by other protein deficiencies. Principle categories typically include mucopolysaccharidoses, mucolipidoses, sphingolipidoses, glycoproteinoses/ oligosaccharidoses, glycogen storage disease, disorders of neutral lipids, and disorders of

protein transport or trafficking (Pastores 2010, Neufeld, Lim & Shapiro 1975, Wraith 2002). Oligosaccharidoses mainly occur when N-linked glycan chains primarily found on glycoproteins are not properly degraded due to the deficient activity of a particular lysosomal acid hydrolase, leading to the irregular accumulation of undigested oligosaccharides and glycopeptides within lysosomes. The degradation process is normally achieved by the sequential removal of terminal residues by a series of lysosomal enzymes and the released monomeric units are transported out of the lysosome (Warner, O'Brien 1983, Cantz, Ulrich-Bott 1990, Thomas 2001).

To complicate matters, since similar glycan structures are also found on macromolecules other than glycoproteins, this classification system can become ambiguous for some LSDs. For example, in G_{M1} and G_{M2} Gangliosidoses, the storage of oligosaccharides may derive from glycolipids and glycoproteins. This will occur because the deficient enzyme can cause the same linkages in all glycoconjugates to be affected (Futerman, van Meer 2004). Therefore, substrate storage may include a complex pattern of oligosaccharide structures (Thomas 2001, Aronson, Kuranda 1989, Michalski, Klein 1999). Table 1 (p.3) describes some common oligosaccharide storage diseases and their corresponding exoglycosidase deficiencies and the accumulating substrates involved in disease progression.

Table 1. Oligosaccharidoses and relevant enzyme deficiency and substrate involved.

Disease	Stored Substrate	Enzyme Deficiency
Sialidosis	Sialyloligosaccharides	α-Neuraminidase
α-Mannosidosis	α-Mannoside	α-Mannosidase
β-Mannosidosis	β-Mannoside	β-Mannosidase
α-Fucosidosis	α-Fucoside	α-Fucosidase
Schindler/Kanzaki disease	α-N-Acetylgalactosaminide	α-N-Acetylgalactosaminidase
Aspartylglucosaminuria	Aspartylglucosamine	Aspartylglucosaminidase
G_{M1} Gangliosidosis (Morquio B disease)	G_{M1} Gangliosides, oligosaccharides, keratan sulfate, glycolipids	β-D-Galactosidase
G_{M2} Gangliosidosis (Tay-Sachs) (Sandhoff disease)	G_{M2} Gangliosides, glycolipids, oligosaccharides	β-D-Hexosaminidase A β-D-Hexosaminidase B

(Adapted from Pastores, 2010)

1.1.2 Clinical presentation, diagnosis and therapeutics

Oligosaccharidoses may present at any age from birth to late adulthood with a wide spectrum of clinical expressions (Table 2; p.6) (Pastores 2010, Wraith 2002, Heese 2008). Most affected individuals appear normal at birth, but depending on the severity of the particular enzyme deficiency, the nature of the storage product, rate of substrate accumulation, and tissue distribution, progressive deposits of incompletely degraded oligosaccharides will cause each patient to display unique molecular, pathological and clinical features (Sheth et al. 2004, Staretz-Chacham et al. 2009). Often times, genetic background and environmental conditions also influence the severity and extent of disease, and genotype and phenotype correlations do not always hold. Patients with identical genotypes, even within the same families, can have significantly different phenotypes (Reuser, Drost 2006). Some common clinical features suggestive of an Oligosaccharidosis include hydrops fetalis, central nervous system dysfunction, coarse facial features, bone abnormalities, organomegaly, ataxia, seizures, weakness, dementia and abnormal eye findings (Pastores 2010, Hwu, Chien & Lee 2010). However, due to the similarity in clinical presentation amongst the Oligosaccharidoses, it is often impossible to identify which specific enzyme or gene is involved in a particular type of storage disease without further biochemical or molecular testing. Consequently, clinical diagnosis can be considerably delayed and sometimes might even be missed (Pastores 2010). As a result, families may have more children before the first affected child is diagnosed and this can become a dilemma for both parents and clinicians.

Once diagnosed, specific and supportive treatments must be considered and implemented. To date, there is no cure for any of the Oligosaccharidoses, but various

therapies are available to treat symptomatic complications and to address the biochemical cause for each particular disease to increase patient survival. Presently, two enzyme delivery systems, hematopoietic stem cell transplantation and intravenous recombinant enzyme replacement therapy, are available (Wenger, Coppola & Liu 2003, Desnick 2004, Sakuraba et al. 2006). Newer therapeutic options under investigation include gene therapy and use of small molecules that inhibit substrate synthesis or serve as a pharmacologic chaperone (Heese 2008, Pastores, Barnett 2005, Beck 2007). Reversibility of symptoms after therapy largely depends on the type of disease and stage of cellular pathology. Ideally, earlier identification and treatment in the neonatal or presymptomatic stage are preferable for successful engraftment and improving overall outcome and quality of life (Meikle, Hopwood 2003, Wilcox 2004).

Currently, the most practical way to identify affected patients before the onset of irreversible pathology is through a newborn screening program. The aim of newborn screening is to detect neonates with genetic disorders that are treatable to facilitate early intervention and to avoid complications before the onset of disease (Fletcher 2006, Kaye et al. 2006, Sahai, Marsden 2009, Wilcken, Wiley 2008). Multiplexing strategies for population and newborn screening for certain LSDs are recently under consideration (Millington 2005, Wilcken 2007, Millington 2008, Marsden, Levy 2010). However, at this moment, there are no multiplex diagnostic methods specifically designed for the group of Oligosaccharidoses. It will be particularly beneficial to develop a multiplex assay to primarily assist clinical diagnosis of Oligosaccharidoses using techniques that will also support future implementation into a newborn screening program.

Table 2. Oligosaccharidoses and clinical manifestations.

Disease	Gene name and location	Age of onset	Clinical manifestations
Sialidosis	NEU1 6p21.3	0-25y	Cherry-red spots, myoclonus, congenital ascites, coarse facial features, hepatosplenomegaly, spasticity, delayed mental and motor development, hydrops fetalis
α-Mannosidosis	MAN2B1 19p13.2-q12	3mon-4y	Mental retardation, delayed speech, cerebellar ataxia, dystosis multiplex, hepatosplenomegaly, hearing loss, hydrops fetalis
β-Mannosidosis	MANBA 4q22-25	1-6y	Mental retardation, hypotonia, spasticity, neuropathy, speech impairment, hydrops fetalis
α-Fucosidosis	FUCA1 1p34.1-36.1	3mon-2y	Progressive motor and mental deterioration, growth retardation, coarse facial features, spasticity, recurrent sinus and pulmonary infections, hydrops fetalis
Schindler/Kanzaki disease	NAGA 22q13.1-13.2	1-4y	Mental retardation, spasticity, myoclonus, angiokeratoma corporis diffusum, hydrops fetalis
Aspartylglucosaminuria	AGA 4q34-35	1-5y	Progressive mental retardation, delayed speech and motor development, coarse facial features, hydrops fetalis
G_{M1} Gangliosidosis (Morquio B disease)	GLB1 3p21.33	6mon-30y	Psychomotor deterioration, dystosis multiplex, hepatosplenomegaly, facial dysmorphism, skeletal dysplasia, hypotonia, hydrops fetalis
G_{M2} Gangliosidosis (Tay-Sachs) (Sandhoff disease)	HEXA 15q23-4 HEXB 5q13	2-6y	Ataxia, psychomotor deterioration, spasticity, coarse facial features, myoclonus, delayed metal and motor development, hydrops fetalis

(Adapted from Pastores, 2010)

1.2. Glycoprotein metabolism

1.2.1 Importance of glycan components on glycoproteins

Glycoproteins are complex macromolecules composed of a polypeptide backbone with covalently attached oligosaccharide chains (Thomas 2001, Taylor, Drickamer 2006). There exist many different types of glycoproteins and they are abundant both within cells and on cell surfaces. Glycoproteins encompass several important classes of macromolecules including enzymes, hormones, immunoglobulins, cell adhesion molecules, structural proteins, transport proteins, toxins, and lectins. Oligosaccharides are integral parts of these macromolecules and greatly affect their physical properties such as conformational stability, viscosity, solubility, resistance to proteolysis, and signal recognition on cell surfaces. This gives rise to glycoprotein's diverse biological functions which include enzymatic catalysis, hormonal control, immunological protection, blood clotting, structural support, cell adhesion, ion transport, cell surface receptor and more importantly allow protein targeting and cell-cell interaction (Taylor, Drickamer 2006, Paulson 1989).

1.2.2 Glycoprotein synthesis

Normal synthesis and turnover of glycoproteins is necessary to maintain overall cellular homeostasis (Winchester 2005). The glycosylation pathways occur in the cytosol, endoplasmic reticulum (ER), and the Golgi complex and involve transport steps, processing glycosidases and glycosyltransferases (Taylor, Drickamer 2006, Durand, Seta 2000, Vellodi 2005). Different pathways generate two main classes of glycans found on glycoproteins (Figure 1; p.10). Oligosaccharides can covalently link to proteins either through oxygen found on a serine (Ser) or threonine (Thr) residue or nitrogen found on an

asparagine (Asn) residue, hence the names O- or N-linked oligosaccharides, respectively. Synthesis of O- or N-linked glycans mainly involve six types of monosaccharides linked in one of two anomeric configurations: β-N-acetylglucosamine (β-GlcNAc), α/β-N-acetylgalactosamine (α/β-GalNAc), α/β-galactose (α/β-Gal), α/β-mannose (α/β-Man), α-fucose (α-Fuc) and sialic acid (α-Sia) (Taylor, Drickamer 2006, Varki 1999, Freeze 2009).

The synthesis of O-linked oligosaccharides begins in the cis Golgi with the transfer of the first sugar residue, GalNAc, to a Ser or Thr residue found on a complete polypeptide chain by a specific polypeptide O-GalNAc transferase. The glycan chain then grows by the addition of various monosaccharide residues in the medial Golgi. O-glycans can range in size from 1 to more than 20 sugars, displaying considerable structural diversity. Moreover, these oligosaccharides are not uniformly distributed along the polypeptide (Taylor, Drickamer 2006, Freeze 2009).

N-linked oligosaccharide synthesis differs from O-linked oligosaccharide synthesis in that N-linked oligosaccharides are built on a dolichol scaffold in the ER prior to being transferred to an Asn residue found in a tripeptide consensus sequence, Asn-X-Ser/Thr (X, any amino acid except proline) on the nascent protein. In contrast, O-linked oligosaccahrides are built on the glycoprotein itself within the Golgi (Taylor, Drickamer 2006, Varki 2009). The biosynthesis of N-glycans begins in the ER with a large precursor oligosaccharide that contains 14 sugar residues. The inner five residues constitute the core, the structure which is conserved in all N-linked oligosaccharides. The precursor is linked to dolichol pyrophosphate, which acts as a carrier for the oligosaccharide. This lipid-linked oligosaccharide is then transferred to an Asn or lysine (Lys) residue on the growing polypeptide chain. The maturation of N-oligosaccharides takes place in the Golgi complex.

This pathway involves a coordinated and sequential set of enzymatic reactions, which remove and add specific sugar residues to the pentasaccharide core (Durand, Seta 2000, Kornfeld, Kornfeld 1985, Medzihradszky 2005, Suzuki, Funakoshi 2006). Although N-linked glycans are more common than O-linked glycans in mammalian glycoproteins, a single glycoprotein may have multiple glycan chains, each containing a mixture of N-linked and O-linked oligosaccharides (Thomas 2001).

N-glycans are usually classified into three main categories based on the differing peripheral branches found on the pentasaccharide core. These are the high mannose type, complex type and hybrid type (Figure 1; p.10). High mannose oligosaccharides have two to six additional mannoses linked to the pentasaccharide core. Complex type oligosaccharides have two or more branches, each containing at least one GlcNAc, one Gal, and eventually one sialic acid. Hybrid oligosaccharides contain one branch that has the complex type structure and one or more high mannose type branches. Complex N-linked oligosaccharides are the predominant types found in serum glycoproteins and are most frequently metabolized by the cell (Taylor, Drickamer 2006, Schachter 1991).

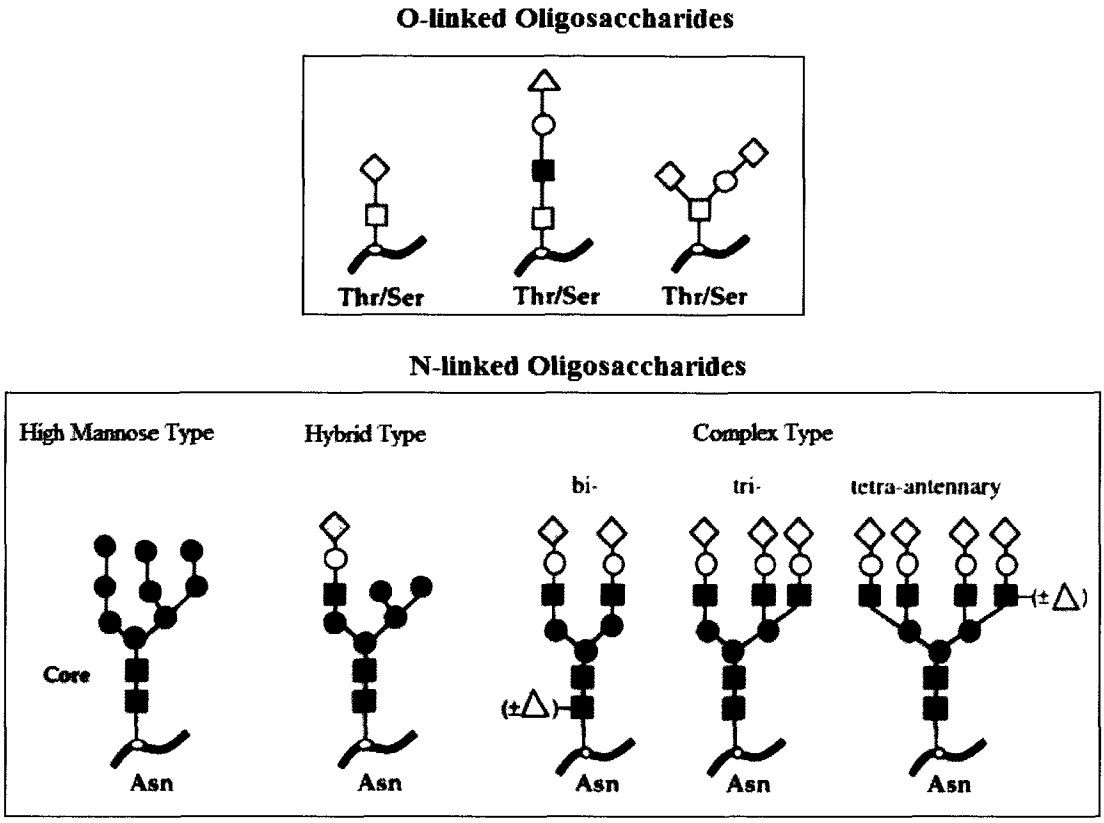


Figure 1. O- and N-linked oligosaccharide structures commonly found on glycoproteins.

■ GlcNAc; ■ GalNAc; ○ Galactose; ● Mannose; Δ Fucose; ◈ Sialic Acid; ~ Polypeptide. The three predominant subgroups of N-linked oligosaccharides include the high mannose type, hybrid type and complex type. The structure within the grey box is the pentasaccharide core common to all N-linked oligosaccharides. (Adapted from Durand et al, 2000)

1.2.3 Glycoprotein degradation

When the modified glycoproteins are degraded, they are targeted to the lysosome by various mechanisms such as endocytosis from outside of the cell or by autophagocytosis from within the cell (Taylor, Drickamer 2006, Freeze 2009, Abraham et al. 1983, Winchester 2005). Lysosomes, which contain the complete array of glycosidases and proteases, constitute the major catabolic organelle for glycoprotein degradation (Winchester 2005). Once inside the lysosome, catabolism occurs predominantly through the combined action of proteases and glycosidases. Proteases act on the protein portion to produce a mixture of amino acids while glycosidases break down oligosaccharides into monosaccharides. Proteolysis occurs from both ends and at internal sites along the polypeptide, unlike the degradation of glycan chains, which is accomplished by the ordered removal of sugars starting at the non-reducing terminal end (Figure 2; p.13) (Thomas 2001, Strecker, Michalski & Montreuil 1988, Aronson, Kuranda 1989). The rate of glycoprotein degradation is unknown; however, it likely depends on the structure and complexity of the protein and glycan chains. It has been found that a large portion of the polypeptide is degraded first before glycan catabolism begins (Freeze 2009). In contrast to N-oligosaccharides, the catabolism of O-oligosaccharides has not been studied so intensively (Winchester 2005, Taylor, Drickamer 2006, Freeze 2009).

There are approximately 50-60 hydrolases found in the lysosome that are involved in the degradation of various macromolecules (Freeze 2009). Most of the glycosidases involved in the degradation of N-glycans are exoenzymes, meaning they must catalyze glycosidic bonds sequentially for the continual and complete breakdown of the oligosaccharide chains. More specifically, catabolism of N-glycans begins with removal of

all peripheral fucose residues by α-Fucosidase. Glycosylaspariginase (Aspartyl-N-acetylglucosaminidase) then cleaves between GalNAc and Asn at the reducing end. Degradation of the remaining free glycan chain occurs in order from the non-reducing end of the molecule until the last monosaccharide at the reducing end is reached (Figure 2; p.13). The exoglycosidases involved in the catabolism of N-linked oligosaccharides are α-Neuraminidase (sialidase), β-D-Galactosidase, β-N-Acetylhexosaminidase, α-D-Mannosidase, β-D-Mannosidase, and α-L-Fucosidase (Taylor, Drickamer 2006, Freeze 2009, Winchester 2005, Strecker, Michalski & Montreuil 1988, Aronson, Kuranda 1989). In an enzyme deficient state, the inability to remove terminal sugar residues causes oligosaccharide accumulation in the lysosome. A specific Oligosaccharidosis is associated with genetic defects in any of these enzymes (Cantz, Ulrich-Bott 1990, Thomas 2001, Durand, Seta 2000).

Of note, the lysosomal system is actually not the only cellular machinery for the breakdown of glycoproteins (Winchester 2005). During biosynthesis, incorrectly folded glycoproteins rejected by the proper ER control mechanism are conjugated to ubiquitin and targeted to the proteosome for degradation of the polypeptide. End products, such as the glycan components remaining in the ER and cytoplasm, will be delivered to the lysosomes for degradation, suggesting another route of glycan delivery to the lysosome (Taylor, Drickamer 2006). This means the structures of some of the oligosaccharides causing diseases are not all digestion intermediates in the lysosomal catabolic pathway but may correspond to intermediates in the biosynthetic pathway, thereby contributing to the broad heterogeneity of oligosaccharides found in the lysosome.

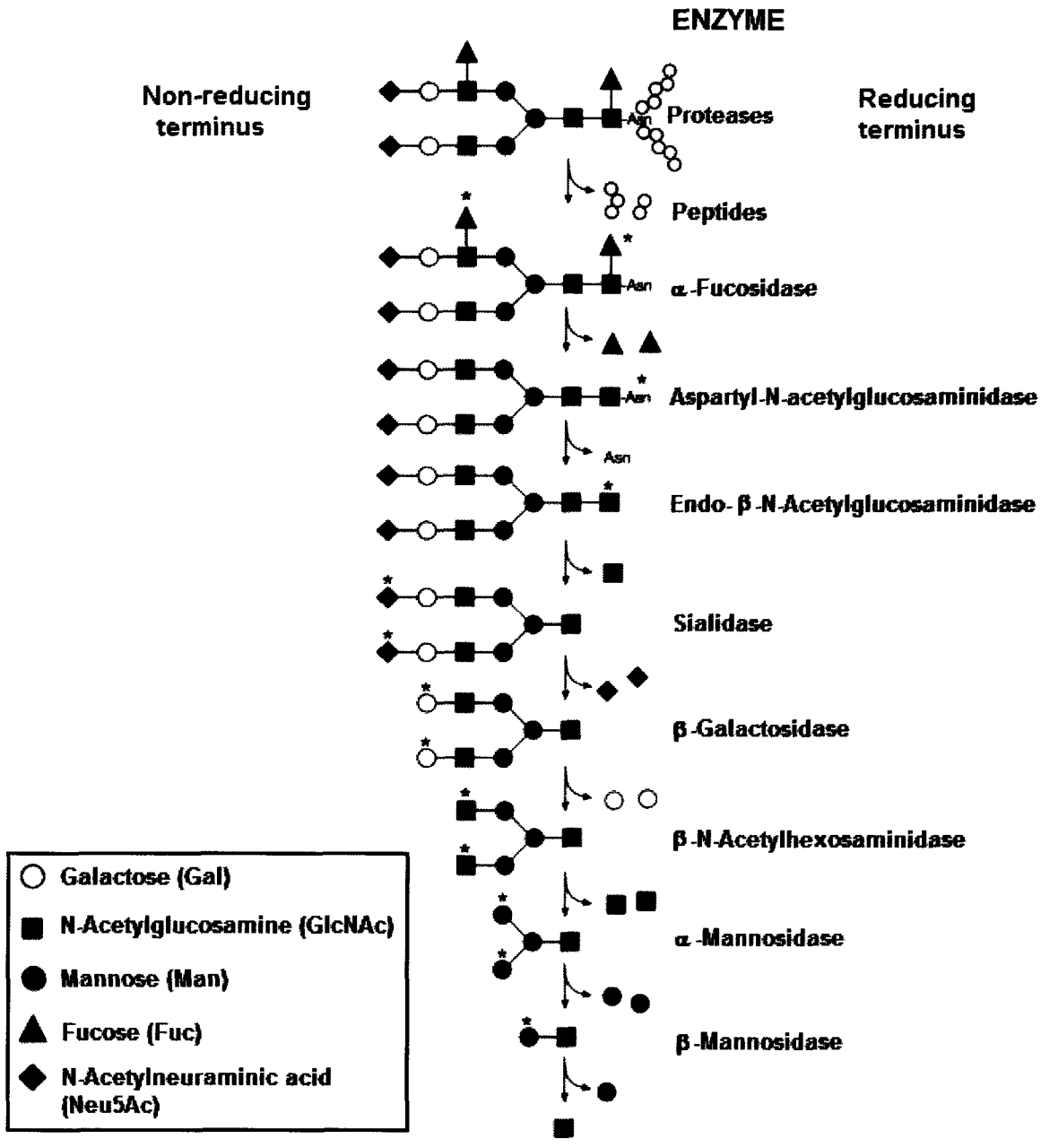


Figure 2. Bidirectional catabolic pathway for complex N-glycans.

Degradation of complex N-linked glycoprotein is bidirectional with the sequential removal of monosaccharides from the non-reducing end by exoglycosidases and proteolysis of the carbohydrate polypeptide linkage at the reducing end by proteases. (Adapted from Freeze, 2009)

1.2.4 The lysosome and exoglycosidase trafficking

Lysosomes are intracellular organelles that belong to the endosomal-lysosomal system, which is responsible for the trafficking, digestion and recycling of endocytosed molecules (Figure 3; p.16). Internalized materials enter endosomal structures first and eventually reach the lysosome where macromolecules are degraded into monomeric subunits (Futerman, van Meer 2004, Vellodi 2005, Karageorgos et al. 1997). An energy dependent proton pump is present in the lysosomal membrane to maintain an acidic environment (pH 4.0-5.5) inside the lysosome and to facilitate the processing of different macromolecules by the acid hydrolases, which are only active at a low pH (Winchester 2005, Vellodi 2005, Fukuda 1991). Lysosomes are capable of fusing with the plasma membrane to secrete contents outside of the cell by carrier-mediated transport systems found on the lysosomal membrane. In glycoprotein catabolism, the amino acid and monosaccharide end products travel between the lumen of the lysosome and the cytosol. Depending on the circumstances, they are either reused by the cell or eliminated from the body. Monomeric sugar units released from glycan chains are the only products reusable by the cell. Partially degraded oligomers cannot reassemble into new polymers and cannot be further metabolized for energy. Accumulation of these oligosaccharide fragments will become toxic to cells and eventually cause cell death (Neufeld, Lim & Shapiro 1975, Durand, Seta 2000, Vellodi 2005). In the diseased state, lysosomes can increase in size and number and account for anywhere between 1% and as much as 50% of the total cellular volume (Freeze 2009, Karageorgos et al. 1997).

Exoglycosidases themselves are actually glycoproteins that are synthesized as inactive precursors in the ER. As with most lysosomal acid hydrolases, they are targeted to

the lysosome via the Mannose-6-Phosphate (Man-6-P) pathway. As they translocate through the ER, maturation occurs and it involves N-glycosylation and loss of the N-terminal signal sequence (Cantz, Ulrich-Bott 1990, Vellodi 2005). In the Golgi apparatus, high mannose type N-linked oligosaccharides on newly synthesized exoglycosidases acquire a phosphate group on a specific mannose residue. The resulting Man-6-P ligand then acts as a specific recognition marker for the binding of exoglycosidases to Man-6-P receptors located in the Golgi apparatus. The Man-6-P marker is the key to separate glycoproteins destined for the lysosome versus those that are secreted. The receptor-enzyme complex then translocates from the Golgi through to the endosomes and finally reaches the lysosomal compartment where the complex is dissociated due to the low pH. The Man-6-P receptors are returned to the Golgi apparatus to transport another exoglycosidase into the lysosome (Winchester 2005, Vellodi 2005, Freeze 2009, Fukuda 1991). Once inside the lysosome, exoglycosidases become active under the acidic environment and begin the catabolic pathway when oligosaccharide containing macromolecules are present.

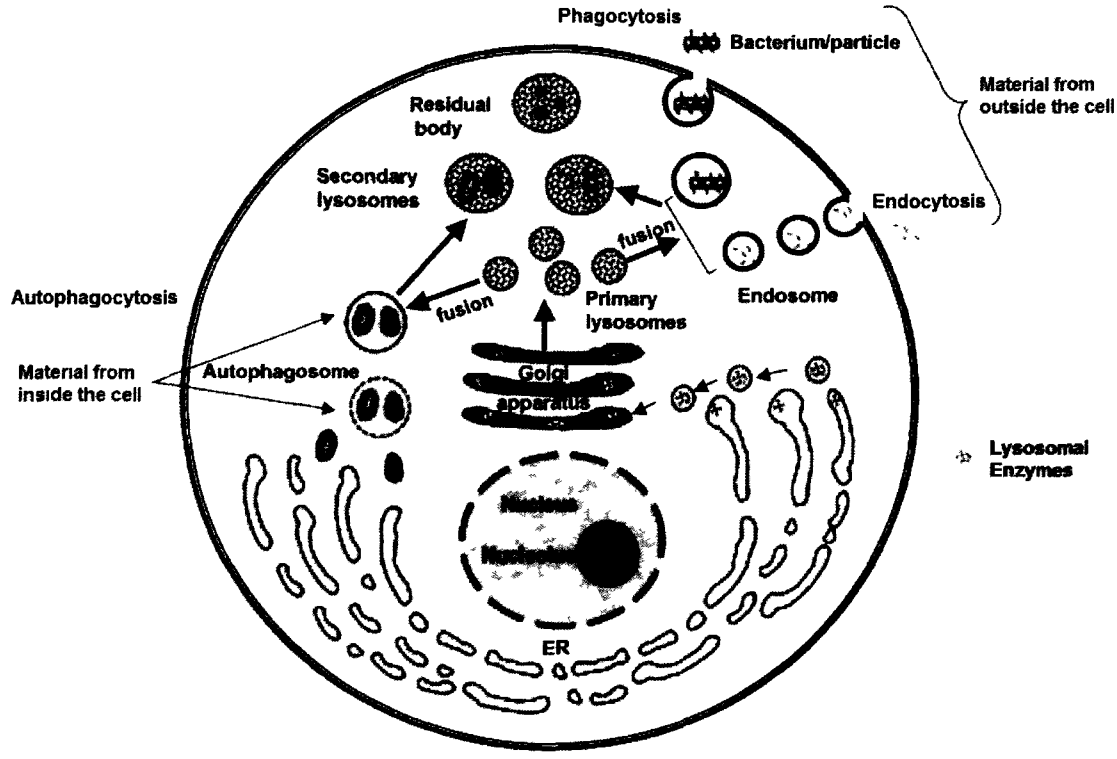


Figure 3. Transport of macromolecules to the lysosome.

The materials to be broken down may enter the cell extracellularly by endocytosis and phagocytosis or intracellularly by autophagocytosis. A series of endosomes and autophagic vesicles transport the contents to the lysosome for degradation. The cargo is delivered when the membranes between the vacuoles fuse. Lysosomal enzymes are synthesized in the ER. After acquiring a Man-6-P marker in the Golgi apparatus, the enzymes are targeted to the lysosome for substrate catabolism to occur. The products return to the cytosol to function as new building blocks for synthesizing new macromolecules. In LSDs, absence of specific enzyme activity in the lysosome leads to improper break down of certain macromolecules, which then accumulate in the lysosome. (Adapted from Vellodi 2005)

1.3 Current Oligosaccharidoses diagnostic methods

1.3.1 Detection of storage materials

For many years now, analyses of urinary oligosaccharides by thin layer chromatography (TLC) (Schindler, Kanzaki & Desnick 1990, Blau et al. 2008) or by high performance liquid chromatography (HPLC) (Hommes, Varghese 1991, Peelen, de Jong & Wevers 1994) have been the primary tests used to screen patients suspected to have an Oligosaccharidosis. The deficiency of one exoglycosidase can lead to the accumulation of undegraded oligosaccharides in lysosomes and increased urinary excretion of carbohydrate material released by the cytolysis of sick cells (Durand, Seta 2000, Sewell 1980).

These methods have several limitations. First, one major obstacle for diagnosis is the lack of specific oligosaccharide biomarkers, which results in complicated oligosaccharide patterns due to the catabolism of different molecules (Ramsay et al. 2005). Additionally, when large volumes of urine containing low creatinine levels are lyophilized, chromatographic quality is reduced since samples will have high salt concentrations. This will give rise to streaking and poor resolution, particularly in TLC, which affects visualization of oligosaccharide patterns (Blau et al. 2008). Moreover, the neonatal period is most susceptible to ambiguous results. On one hand, newborns with an Oligosaccharidosis can appear normal at birth and show normal urine oligosaccharides because many of the toxic metabolites can be cleared by the mother during gestation (Staretz-Chacham et al. 2009). On the other hand, breast-fed neonates and infants fed with fortified milk often show increased oligosaccharide excretion patterns that are not a true reflection of the oligosaccharide profile in normal newborn urine (Pastores 2010).

Therefore, urinary analysis almost always requires confirmation by more definitive diagnostic methods such as mutation or enzyme analysis.

1.3.2 Enzymology

Demonstrating enzyme deficiency is currently the most efficient way to diagnose Oligosaccharidoses and other LSDs. Two different types of enzymatic methods have been recently proposed and these involve protein quantification or measurement of enzyme activity in plasma, leukocytes or cultured skin fibroblasts. In both methods, the basic concept is that in most LSDs, a genetic mutation will result in alterations causing a decrease in the enzyme, an absence of the enzyme or a deficiency in enzyme activity, as compared to wild type controls. This may result from RNA instability leading to poor protein translation, a premature stop codon that causes improper protein synthesis leading to proteosomal degradation, or the protein half-life in the lysosome may simply be shorter. Other factors that could affect enzyme activity but not protein abundance include enzyme active site mutations or improper trafficking of the enzymes to the lysosome due to transport protein mutations (Staretz-Chacham et al. 2009).

In protein quantification, taking advantage of the decrease in protein level has led to the development of an immune-capture quantification assay, which provides protein abundance data based on fluorescence detection methods. Relative to the controls, differences in the concentrations of particular protein markers are used to diagnose specific LSDs (Meikle et al. 1997, Umapathysivam et al. 2000). These immunological assays can be multiplexed, but determination of protein abundance data is not always useful, especially when non-functional proteins without catalytic activity are produced. In fact, measuring protein abundance may result in affected patients that demonstrate erroneously

normal enzyme levels (Meikle et al. 2006). Another limitation of this method is the labour-intensive procedures of preparing purified antibodies against the specific protein markers. New protein markers are still under investigation for proper diagnosis of individual LSDs, but currently this method is not available for the group of Oligosaccharidoses (Wenger, Coppola & Liu 2003).

A more precise and practical method to diagnose each Oligosaccharidoses is by determining the activity of the relevant exoglycosidase present in a biological sample. This is accomplished by measuring the amount of 4-methylumbelliferyl (4MU) product released from an artificial substrate that is composed of a specific monosaccharide linked to a 4MU molecule. The glycosidic bond is only recognized and cleaved by a target exoglycosidase. The amount of liberated 4MU is measured by fluorescence spectroscopy and the amount of enzyme activity can be calculated (Blau et al. 2008). Of note, the previous immune-capture quantification approach had been further modified to detect enzyme activity using 4MU substrates in this manner. Specific enzymes that are captured from the immunoassay are subsequently incubated with a target 4MU substrate to determine their activities (Umapathysivam, Hopwood & Meikle 2001). However, the method used for collecting pure enzymes still involves the use of antibodies which is quite cumbersome and time-consuming to prepare. More recently, an enzyme source containing various proteins collected directly from a biological sample such as plasma, leukocytes or cultured skin fibroblasts has been conveniently used for the *in vitro* 4MU-based exoglycosidase endpoint assays to determine enzyme activities. The main drawback with this approach is that each assay uses the same indicator, 4MU, for measuring accumulating product, so multiplexing is prohibited.

1.3.3 Molecular genetic testing

Oligosaccharidoses and other LSDs are monogenic disorders, typically inherited in an autosomal recessive manner. The genes for most Oligosaccharidoses have been characterized (Table 2; p.6), enabling rapid identification of potential disease-causing mutations. However, molecular genetic analysis is usually not the method of choice to diagnose or confirm suspected clinical cases of Oligosaccharidoses. This is due to the higher costs involved in testing and many of the disease-causing mutations are generally private, where different mutations in different pedigrees can lead to the same disorder, and relatively few public mutations are described. Such mutations can be missense, nonsense and splice-site mutations, or partial deletions and insertions (Pastores 2010, Wenger, Coppola & Liu 2003). However, finding a novel deviation from a consensus sequence does not necessarily mean the mutation is disease-causing. The possibility of identifying new variants of unclear significance actually makes this an even less sensitive detection method. Furthermore, there are generally no direct correlations between genotype and phenotype among these disorders. Genotype alone cannot predict the clinical course of these disorders (Thomas 2001, Marsden, Levy 2010).

1.4 Developments in LSD multiplex assays

1.4.1 Newborn screening history

Phenylketonuria (PKU), with a prevalence of 1 in 10,000 births, was the first IEM screened in newborns (Wilcken 2007, Wilcken, Wiley 2008). Due to a deficiency of hepatic phenylalanine hydroxylase, phenylalanine (Phe) builds up in the blood and severe mental retardation can occur. Infants with PKU can develop normally by early implementation of a low Phe diet. In the 1960s, Guthrie et al. developed a simple bacterial inhibition assay to monitor blood Phe concentrations allowing early identification of PKU (Guthrie, Susi 1963). This test was refined to require only a few drops of newborn blood collected by the heel stick method onto filter paper, which easily dries at room temperature. Dried blood spots (DBS) have since been used as the principle sample type in newborn screening laboratories which significantly improved previous sample collection, transportation and storage methods (Chace et al. 1999, Mei et al. 2001, De Jesus et al. 2009). By the 1990s, Millington et al. proposed the use of tandem mass spectrometry (MS/MS), a device that can separate and quantify multiple analytes based on their mass to charge (m/z) ratios, to detect various metabolites in DBS (Millington et al. 1990). In collaboration with Chace et al., a new and more efficient PKU diagnostic method was developed, which involved detecting Phe from DBS using MS/MS (Chace et al. 1993, Chace et al. 1998). Today, more than 30 IEM, including amino acidemias, organic acidemias, and disorders of fatty acid oxidation can be detected by MS/MS (Sweetman 1996, Levy 1998, Sansom 1999, Zytkovicz et al. 2001, Schulze et al. 2003, Wilcken et al. 2003, Wiley et al. 2003). Identifying IEM in DBS using MS/MS is now a routine clinical practice leading to earlier detection and improved outcomes (Clarke 2002, Chace, Kalas &

Naylor 2002, Chace, Kalas & Naylor 2003, Sahai, Marsden 2009, Pitt 2010) Often, more cases are detected by newborn screening than by clinical presentation (Chace, Kalas 2005).

1.4.2 LSD diagnosis using DBS

The late Nestor Chamoles and his coworkers were the first group to show that lysosomal enzyme activities could be measured *in vitro* using 4MU substrates by eluting enzymes in DBS from a filter card; the released 4MU fluorogenic product could be detected by fluorescence spectroscopy (Chamoles, Blanco & Gaggioli 2001a, Chamoles, Blanco & Gaggioli 2001b, Chamoles et al. 2001, Chamoles et al. 2002b, Chamoles et al. 2002a, Chamoles et al. 2004). These assays had been employed together to screen for several LSDs at the same time, but reactions needed to be carried out separately since 4MU was the common product for each enzyme analysis (Civallero et al. 2006).

More recently, the use of MS/MS as a detection technique to measure enzymatic products for the evaluation of individual exoglycosidase activities within a small sample of DBS had been examined for the diagnosis of several LSDs (Li et al. 2004a, Wang et al. 2005, Wang et al. 2007, Blanchard et al. 2008). Most of these assays simply involved the addition of synthetic substrates for particular enzymes of interest to a DBS that was rehydrated in a buffer. After incubation, the reaction products were quantified by MS/MS using specific isotope labelled internal standards and the enzymatic activities were calculated. Since MS/MS had the potential to detect multiple analytes based on mass differences in a single sample injection, this technology had been intensively explored to assist the development of a multiplex assay for the group of LSDs.

1.4.3 LSD multiplex assay

In a landmark study conducted by Gelb and colleagues (Li et al. 2004b, Gelb et al. 2006), the group was one of the first to successfully describe a new multiplex assay that can distinguish up to five lysosomal enzyme activities in DBS using the MS/MS technology. LSDs under investigation were selected because treatments are currently available and they include Fabry, Gaucher, Krabbe, Niemann-Pick A/B, and Pompe diseases. This assay initially required dividing a rehydrated DBS extract into separate incubations for the enzymes that target the five diseases. An artificial substrate, closely resembling the natural substrate, was incubated with an aliquot of DBS extract. Depending on the activities of the target enzyme, a set of unique products were expected. Optimal buffer conditions were also presented to mimic the *in vivo* lysosomal environment. Internal standards, either isotope labelled analogs or closely related homologs of the enzyme reaction products, were used for quantification of these products. After recombining the reactions and before the final analysis, liquid-liquid extraction followed by solid-phase extraction with silica gel was used to remove buffer components and other reagents incompatible with the MS/MS assay. All enzymatic products and their internal standards could be resolved by MS/MS in a single analysis because of the differences in mass to charge (m/z) values (Figure 4; p.24) (Kasper et al. 2010). The amount of product was calculated from the ion abundance ratio of the product to the internal standard for a given sample and the enzyme activities were typically determined in units of μmol/h/L blood.

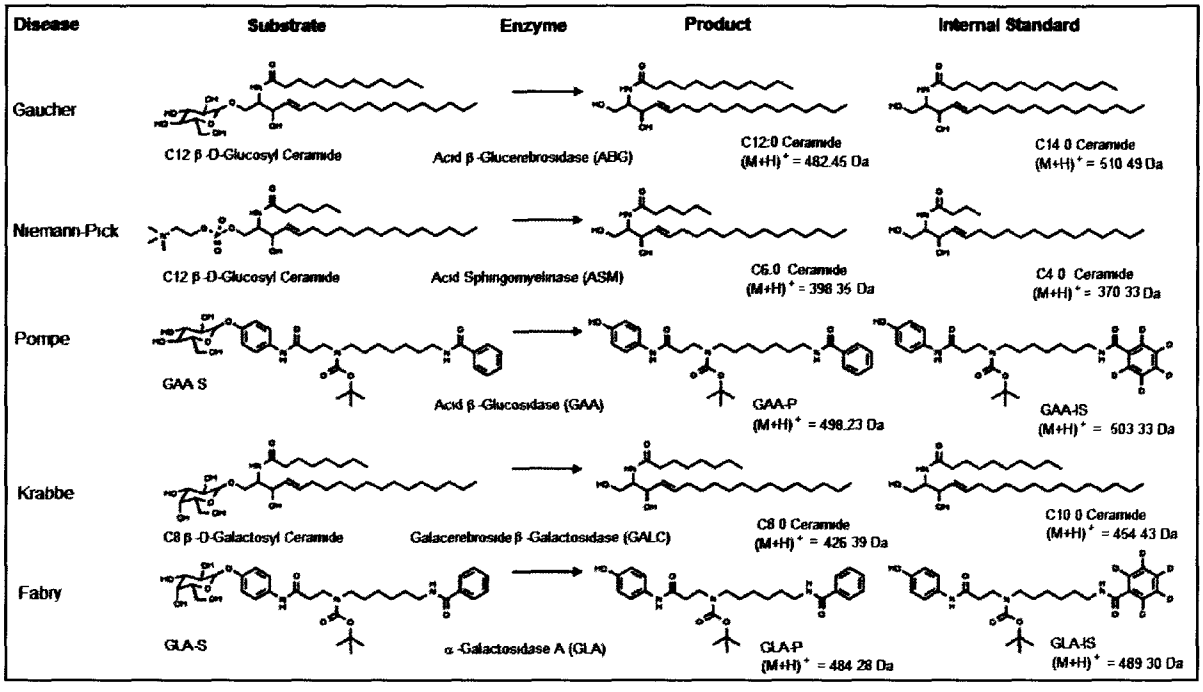


Figure 4. Reaction scheme of a previous multiplex assay screening for treatable LSDs in DBS using MS/MS.

The assay involved the separate incubation of DBS aliquots with optimal buffers containing unique substrates targeting the five lysosomal enzymes of interest. Presence of functional enzymes yielded products that have different masses. Reactions were combined at the end and MS/MS was used to simultaneously measure the five enzymatic products. Internal standards were included in the reaction mixture to assist product quantification and enzymatic activities were subsequently calculated for the diagnosis of the five LSDs. (Adapted from Kasper et al, 2010)

1.5 Thesis Outline

1.5.1 Rationale

MS/MS is a very useful tool in diagnostic and newborn screening laboratories for early detection and confirmation of many inborn metabolic disorders. As described earlier, a multiplex assay for five treatable LSDs was developed recently using DBS and MS/MS by a research group at the University of Washington (Li et al. 2004b, Gelb et al. 2006). This multiplex assay is still under development for eventual implementation into newborn screening programs and more LSDs are currently considered for incorporation (De Jesus et al. 2009, Zhang et al. 2008). To date, only one MS/MS based LSD assay for the diagnosis of Krabbe is routinely tested in a newborn screening program in New York State (Duffner et al. 2009). In Ontario, diagnostic testing for suspected LSD patients, particularly for the Oligosaccharidoses, can only be done in specialized diagnostic centres and most continue to use the traditional 4MU-based fluorescence assay methods. It becomes tedious when multiple assays have to be run in parallel to screen for the deficient enzyme. Furthermore, the sample types used for testing include plasma, leukocytes or fibroblast skin cells, which often require invasive collection techniques (Alroy, Ucci 2006).

At present, the use of MS/MS as a detection technique to measure unique enzymatic products and allow lysosomal enzyme activity determination in DBS has not been explored for the Oligosaccharidoses. Development of such an assay will facilitate diagnosis of individuals suspected to have an Oligosaccharidosis since clinical presentation often does not identify the specific enzyme involved. Such an assay would also be useful for future integration of Oligosaccharidoses into newborn screening programs.

Newborn Screening Ontario (NSO) at the Children's Hospital of Eastern Ontario (CHEO) has implemented several MS/MS assays for the screening of many genetic disorders using DBS. As this new technology is readily available, has low limits of detection capabilities for many analytes over a wide m/z range and has a high degree of analytical specificity, this research aims to develop a MS/MS based assay for the simultaneous measurement of multiple enzymatic products to determine exoglycosidase activities in DBS. The target exoglycosidases under investigation are α-Neuraminidase, β-Galactosidase, β-Hexosaminidase, α-Mannosidase, β-Mannosidase and α-Fucosidase.

1.5.2 Hypothesis

A set of substrates and assay conditions can be identified for target exoglycosidases to allow *in vitro* measurement of different exoglycosidase activities from human tissues in a multiplex assay by quantification of unique products using tandem mass spectrometry (MS/MS).

1.5.3 Objectives

1. Investigate and compare exoglycosidase activities among different biological sample types using previous 4MU-based fluorescence assays.
2. Identify a universal buffer where one *in vitro* enzyme assay environment allows measurement of multiple exoglycosidase activities.
3. Develop a MS/MS based quantitative assay for determining exoglycosidase activities by measuring derivatized oligosaccharide products.
4. Demonstrate a multiplex assay can be established using MS/MS by simultaneously measuring several distinct exoglycosidase products within a single reaction tube.

PART A: Exoglycosidase activities in different human tissues

2.1 Introduction

Diagnosis of LSD traditionally involves assaying the enzyme of interest by means of an artificial substrate with a fluorescent label, such as 4MU, which is linked to a monosaccharide via an α- or β- glycosidic bond that is recognized by a particular enzyme usually derived from serum, leukocytes or cultured skin fibroblasts (Figure 5; p.28) (Alroy, Ucci 2006). More recently, due to the increased ease of obtaining and transporting dried blood samples, fluorescence based enzymatic assays using DBS have been investigated and adapted. Table 3 (p.29) describes a set of 4MU fluorescent based enzyme assays for the individual identification of several Oligosaccharidoses. These methods are currently used by the Child and Parent Resource Institute (CPRI), a specialized regional diagnostic laboratory located in London, Ontario, Canada. Leukocytes and fibroblasts are typically used as the enzyme source. In the current study, these assay conditions were examined individually to compare exoglycosidase activities in leukocytes, fibroblasts and more importantly in DBS.

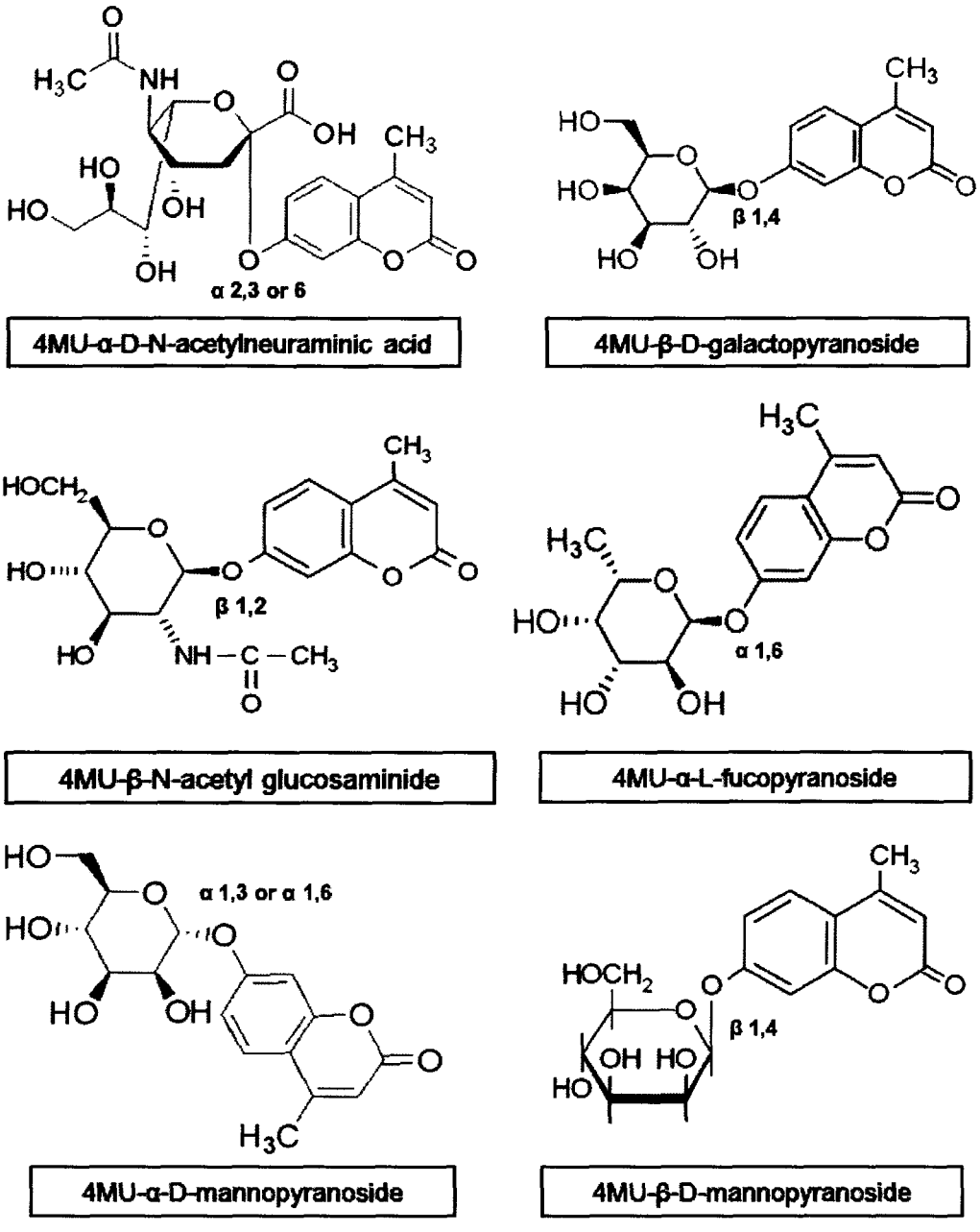


Figure 5. 4MU exoglycosidase substrates.

Each of these substrates contains the 4MU moiety linked to a specific monosaccharide. Specific glycosidic linkages only allow one particular exoglycosidase to cleave the glycosidic bond. These substrates target the exoglycosidases found in the N-linked glycan catabolic pathway.

Table 3. Exoglycosidase 4MU-based fluorescent assays used at CPRI.

Enzyme	4-MU Substrate	Unique Buffer Condition	Reaction Volumes*
α-Neuraminidase (Sialidase)	4.0 mM 4MU-α-D-N-acetylneuraminic acid	0.5 M sodium acetate-acetic acid, pH 4.3	50µl E+ 40µl B+ 20µl S
β-Galactosidase	1.0 mM 4MU-β-D-galactopyranoside	0.2 M lactic acid-sodium hydroxide, pH 3.6	50µl E+ 50µl B+ 200µl S
β-Hexosaminidase	3.0 mM 4MU-β-N-acetyl glucosaminide	0.03 M citrate-phosphate, pH 4.1	50µl E+ 50µl SB
α-Mannosidase	1.0 mM 4MU-α-D-mannopyranoside	0.05 M citrate-phosphate, pH 4.0	20µl E+ 100µl SB
β-Mannosidase	3.0 mM 4MU-β-D-mannopyranoside	0.2 M citrate-phosphate, pH 4.2	20µl E+ 75µl H_2O+ 100µl SB
α-Fucosidase	1.0mM 4MU-α-L-fucopyranoside	0.05 M citrate-phosphate, pH 4.5	20µl E+ 100µl SB

(Adapted from CPRI)

*E-enzyme
B-buffer
S-substrate reconstituted in water
SB-substrate reconstituted in buffer

2.2 Materials and Methods

Unless otherwise specified, all reagents used were of the highest grade commercially available.

2.2.1 DBS preparation for enzyme collection

Blood samples from healthy individuals were spotted (aliquots of 75 µl) on filter paper (Schleicher and Schuell no. 903) and allowed to dry overnight at room temperature. DBS samples were stored at 4°C in plastic bags until analysis. 3.0 mm discs were punched out from a DBS using a Harris Uni-Core hand puncher (Whatman Inc., US) and placed into a 24-well plate. For each 3.0 mm disc, 100 µl of H_2O was used for enzyme extraction. The plate was shaken for 30 minutes at 4°C. Leaving the discs behind, the aqueous portion was collected and used directly for exoglycosidase activity assays.

2.2.2 Leukocyte preparation for enzyme collection

For every 5 ml of freshly drawn blood from healthy individuals, 1 ml leukocyte solution (5 g Dextran T-500, 0.7 g NaCl, 50 mg heparin in 100 ml H_2O) was added. The tube was inverted 10 times to mix the solution. The sample was left to sit at room temperature for 45-60 minutes. The upper portion of the sample was transferred to a 10 ml centrifuge tube and the lower portion was discarded. The top layer was then centrifuged at 450 x *g* at 4°C for 10 minutes and the supernatant was discarded. To the pellet, 0.8 ml of 0.9% (w/v) NaCl and 2.4 ml of H_2O was added. The sample was vortexed at moderate speed for 90 seconds and then 0.8 ml of 3.6% (w/v) NaCl was added. The sample was further spun at 450 x *g* at 4°C for another 10 minutes. This step involving the addition of 0.9% (w/v) and subsequently 3.6% (w/v) NaCl was repeated. The supernatant was

discarded and the leukocyte pellet was either stored at -20°C or further treated for use as an enzyme source. For enzyme collection, 1 ml H_2O was added to the leukocyte pellet and homogenized (~20 strokes) in a 7 ml Kontes Dounce tissue grinder (Fisher Scientific, Canada). The homogenate was centrifuged at 3000 x *g* at 4°C for 10 minutes and the supernatant was collected and used directly for exoglycosidase activity assays. For each leukocyte sample, an aliquot was taken for protein quantification using the Lowry method (Lowry et al. 1951).

2.2.3 Fibroblast cell culture and harvesting of cells for enzyme collection

Normal primary skin fibroblasts used in this study were provided by CPRI. Cells were maintained in 75-cm^2 flasks according to established procedures in Minimum Essential Medium Alpha Modification (1x) (MEM Alpha) with L-Glutamine and Ribo/Deoxyribonucleosides, 5% (v/v) Fetal Bovine Serum (FBS), and Penicillin-Streptomycin antibiotic solution (100x), in a 5% CO_2 atmosphere incubator. At harvesting time when cells reached 90% confluency, medium was removed and the cells were washed with Dulbecco's Phosphate Buffered Saline (1x) (DPBS). The cells were removed by trypsinization (3 ml of 0.05% Trypsin-EDTA 4Na (1x) per flask for 5 minutes at 37°C). Medium was added to stop the trypsin reaction and cells were transferred into a new 10 ml plastic tube. Fibroblasts were spun down at 1000 rpm at 4°C for 10 minutes. Supernatant was removed and 1 ml H_2O was added to the pellet and further broken down by homogenization (~20 strokes) in a 7 ml Kontes Dounce tissue grinder (Fisher Scientific, Canada). Lysate was transferred to a new tube and spun at 3000 x *g* at 4°C for 10 minutes. Supernatant was collected and used directly for exoglycosidase activity assays. An aliquot

of the cell lysate was taken for protein quantification using the Lowry method (Lowry et al. 1951).

2.2.4 Lowry protein quantification method

Protein samples up to 100 µl (containing up to 100 µg protein) were placed into a 10 ml plastic tube. All samples were brought to a total volume of 1 ml with Folin A solution (0.1 M NaOH containing 2% (w/v) Na_2CO_3). 5 ml of Folin B (2% (w/v) potassium sodium tartrate tetrahydrate and 0.6% (w/v) $CuSO_4$ in Folin A) was added to each tube. Samples were vortexed for a few seconds and left at room temperature for 10 minutes. 0.5 ml of Folin C (1:1 H_2O: Folin & Ciocalteu's phenol reagent) was subsequently added, vortexed and left at room temperature for another 30 minutes. Absorbance was read at 750 nm on a spectrophotometer.

2.2.5 4MU fluorescent assays

Exoglycosidase activities from DBS, leukocytes, or fibroblasts, were assayed using current protocols from CPRI (Table 3; p.29). For each exoglycosidase reaction, appropriate amounts of buffer and 4MU substrates were added into a 10 x 75 mm borosilicate glass culture tube. Reactions were initiated when the enzyme source was added and the tube was incubated at 37°C. Reactions were carried out for a defined period of time and stopped by adding 1 ml of Glycine buffer (0.2 M Glycine, 0.125 M Na_2CO_3 and 0.1 M NaCl) at pH 10.7. For the T=0 control sample, the Glycine buffer was added immediately after the enzyme aliquot. For analysis, 200 µl of the reaction was transferred into a 96-well plate. The fluorescence due to the release of 4MU from the substrate by appropriate exoglycosidase activity was measured using a Biotek Synergy 2 microplate fluorescence

reader (Fisher Scientific, Canada). The excitation wavelength used was 365 nm and the emission wavelength was at 450 nm. The fluorescence readings were corrected for blanks, and the results were compared with the fluorescence from a 4MU (4-methylumbelliferone sodium salt) calibrator. A calibration curve was included for each batch of enzyme assays by serial dilution starting with 10 μM 4MU in a Glycine buffer. 4MU standard solutions were light sensitive and were only stable for two weeks time at 4°C.

2.3 Results

2.3.1 Generating a standard curve to test system linearity

The Biotek Synergy 2 system (Fisher Scientific, Canada) was the microplate fluorescence detector used for 4MU analysis in 96-well plates. 4MU was used as a standard calibrator since 4MU was the product released in all the enzyme reactions. Linearity was maintained between 10 and 1500 fluorescence units in this system as shown in Figure 6 (p.34). This calibration assay was performed with every new batch of enzyme experiments to check for reliability and consistency on the standard, the instrument and the technique.

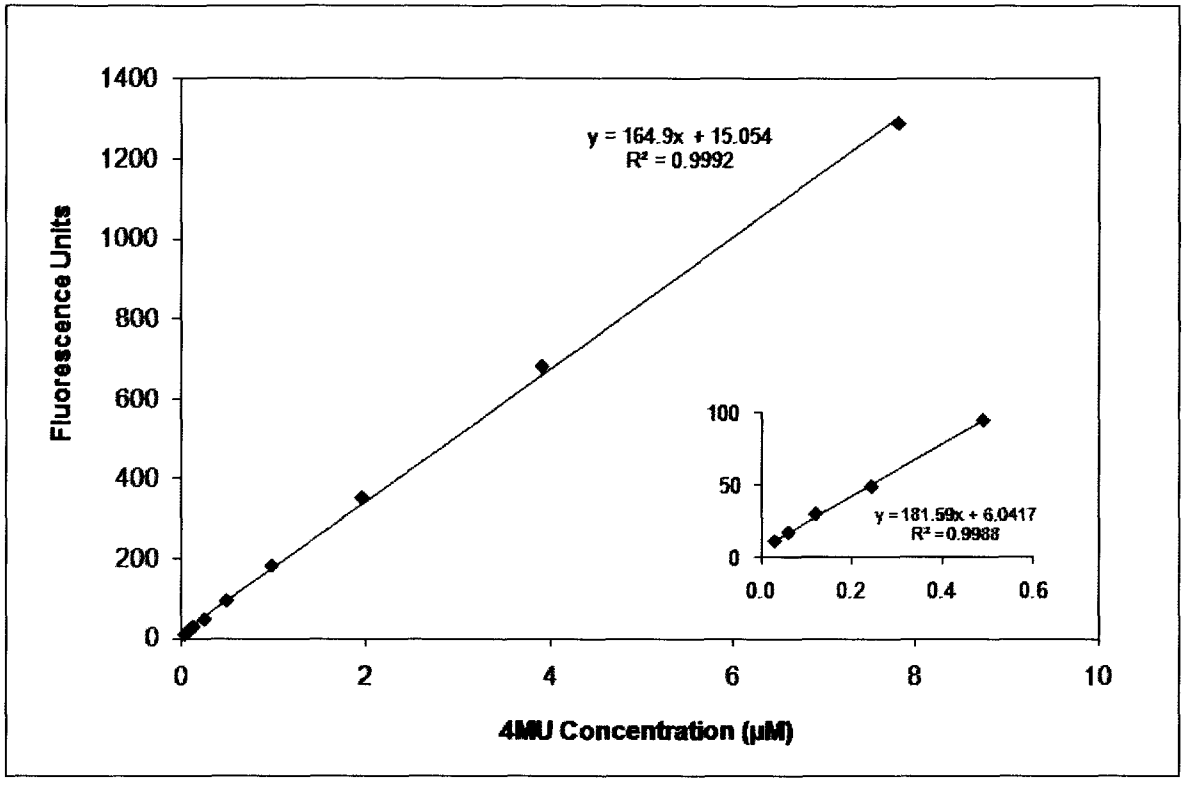


Figure 6. 4MU calibration curve in a fluorometric system.

Instrumentation used was a Biotek Synergy 2 microplate fluorescence reader. Under standard conditions, a linear response was observed over the range of 10-1500 fluorescence units.

2.3.2 Exoglycosidase activities in DBS, leukocytes and fibroblast

Table 4 (p.36) compares the exoglycosidase activities determined in DBS expressed in nmol/h/DBS and in leukocytes and fibroblasts, both expressed in nmol/h/mg protein. Two different individuals were tested for each tissue type and each sample was analyzed in duplicate. Enzymatic activity for β-Hexosaminidase, β-Galactosidase, α-Mannosidase and β-Mannosidase were detectable in all tissue types of interest under the assay protocols provided by CPRI (Table 3; p.29). Fibroblasts demonstrated the highest exoglycosidase activities and DBS gave the lowest exoglycosidase activities, which were only slightly above background. Due to the lack of commercial availability of the 4-MU-α-L-fucopyranoside substrate at the time of study, α-Fucosidase was only determined in leukocytes and fibroblasts. α-Neuraminidase activity was tested in all tissue sample types, but true activity could not be determined as a result of substrate hydrolysis which consistently gave higher fluorescence signal in the water control (4MU-α-D-N-acetylneuraminic acid substrate in water without enzyme) as compared to the actual enzymatic reaction (4MU-α-D-N-acetylneuraminic acid substrate in presence of enzyme), which was run in parallel. From here on, α-Neuraminidase was omitted from further analysis.

Table 4. Exoglycosidase activities in DBS, leukocytes and fibroblasts.

	DBS (nmol/h/DBS)		**Leukocytes** (nmol/h/mg protein)		**Fibroblasts** (nmol/h/mg protein)	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
α-Neuraminidase (Sialidase)	nd	nd	nd	nd	nd	nd
β-Hexosaminidase	0.80 ± 0.009	1.14 ± 0.008	93.08 ± 0.06	123.16 ± 4.24	578.31 ± 3.00	645.92 ± 0.67
β-Galactosidase	0.07 ± 0.004	0.05 ± 0.009	63.66 ± 2.51	14.08 ± 0.21	176.40 ± 1.98	172.51 ± 1.99
α-Mannosidase	0.03 ± 0.017	0.08 ± 0.000	18.70 ± 1.08	23.23 ± 0.15	28.83 ± 0.46	30.12 ± 0.34
β-Mannosidase	0.05 ± 0.052	0.07 ± 0.008	8.12 ± 0.60	19.38 ± 0.44	8.64 ± 1.53	26.11 ± 1.03
α-Fucosidase	nd	nd	6.80 ± 0.14	7.10 ± 0.25	18.17 ± 0.13	19.83 ± 0.59

nd - not detected

Sample1, Sample2 – normal patient samples

Each patient sample was analyzed in duplicate. Results are expressed as the mean ± SE.

2.3.3 Time course analysis of exoglycosidase activities

The effect of variable incubation times (up to 24 hours) on the enzymatic activities from cultured skin fibroblasts and DBS are shown in Figure 7 (p.38). Fluorescence detected within these incubation times, which represents 4MU released, were well within the linear range of the 4MU calibration curve. Samples were analyzed in duplicate for each time point. The extended incubation time was intended to demonstrate the possibility of using DBS as an alternative sample type for these enzymatic assays despite the minimal signal observed in the previous DBS analysis that terminated at 1 hour. In this 24 hour time course study, all exoglycosidases revealed increasing activity with increasing incubation time. However, lysosomal enzymes from fibroblasts, compared to DBS, generally produced more fluorescence in a shorter amount of time. Most enzymes in fibroblasts displayed a hyperbolic trend where a plateau was reached within the first few hours of incubation, likely signifying substrate depletion. As for DBS, more linear curves were obtained. An incubation time of 24 h or more might be necessary when this sample type is used for future assays. Since fibroblasts demonstrated more robust exoglycosidase activities in a shorter amount of time, this was the tissue type used for the remainder of this research study. An incubation time of 2 hours for β-Hexosaminidase and β-Galactosidase and 12 hours for α-Mannosidase, β-Mannosidase and α-Fucosidase were selected for further enzyme assay analysis since it was the highest signal which was found within the linear range.

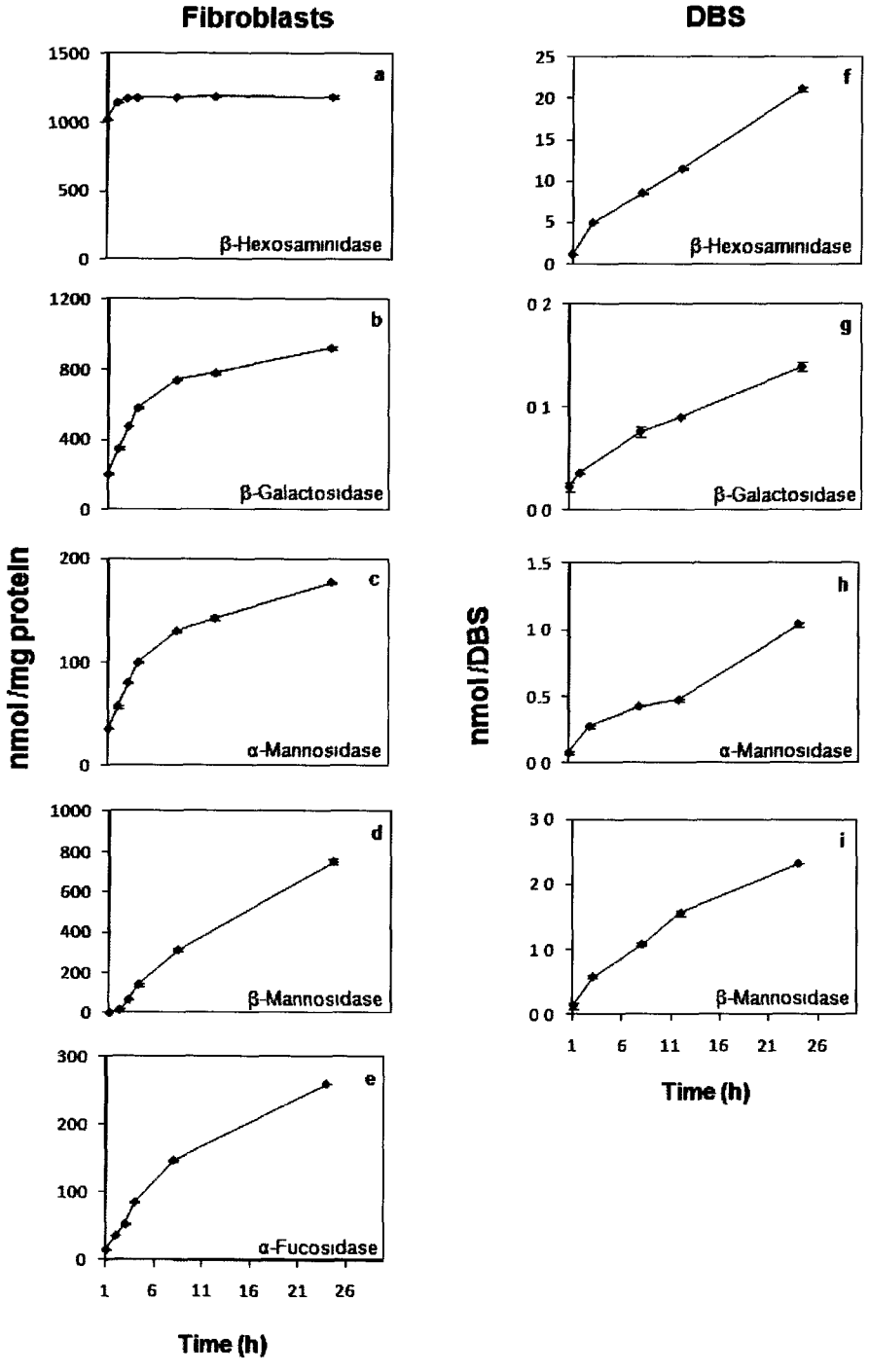


Figure 7. Enzyme activities measured as a function of incubation time.

β-Hexosaminidase (a,f), β-Galactosidase (b,g), α-Mannosidase (c,h) and β-Mannosidase (d,i) were detected from cultured skin fibroblasts and DBS samples. α-Fucosidase (e) was only analyzed in fibroblasts. Results represent the mean ± SE of 2 independent experiments.

2.4 Discussion

Prior to the development of assays involving blood samples on filter paper, enzyme analysis required tissue biopsies (e.g. skin, bone marrow or liver) or whole blood obtained from the patient to be sent to specialized laboratories with the appropriate technologies and expertise for diagnosis of a particular LSD (Alroy, Ucci 2006). Techniques developed using DBS as a starting material may eliminate the need to examine tissues, but this method of testing still remains a subject of continuing validation due to the obvious differences in lysosomal enzyme abundance between DBS and other varying tissue types. In DBS, the enzyme activity is likely plasma and white blood cell derived. However, during the process of drying the blood on the filter paper, many enzymes can become denatured as a result of dehydration. Although earlier studies have shown that lysosomal enzymes are relatively very stable and can withstand drying, the first step in this research was to evaluate the exoglycosidase activity differences in DBS as compared to leukocytes and fibroblasts using the previously described 4MU fluorescence based enzyme assays (Table 3; p.29). Interestingly, β-Hexosaminidase, β-Galactosidase, α-Mannosidase and β-Mannosidase activities were detected in all three tissue types by monitoring the amount of 4MU released under the assay conditions provided by CPRI. As expected, DBS revealed minimal exoglycosidase activities as compared to fibroblasts and leukocytes. For α-Fucosidase, activity was only determined in leukocytes and fibroblasts due to the lack of substrate availability at the time of study. In spite of this situation, α-Fucosidase is predicted to be present in DBS, probably at lower activity than the rest of the exoglycosidases, as observed in leukocytes and fibroblasts. α-Neuraminidase activity could not be determined in any of the sample types, which is anticipated since this enzyme is

known to be acid labile and denatures easily. Fluorescence detected in the α-Neuraminidase water control may have been due to substrate instability and hydrolysis from the aqueous environment. Further studies would need to be done to better understand how this enzyme functions. However, since this was not a primary focus in this project, α-Neuraminidase analysis was disregarded in the rest of this research. Of note, developing a new multiplex assay for the remaining exoglycosidases of interest will still be a very useful test.

The next step was to explore the kinetics of these exoglycosidases and to determine the optimal incubation times to obtain maximum activities for these lysosomal enzymes that are present in the different tissue types. Since collecting enzymes from cultured skin fibroblasts was more convenient than from leukocytes and DBS was the ultimate sample type, only these two tissue types were investigated for comparing exoglycosidase activities over a 24 hour time course. From this experiment, it was found that DBS needed a much longer incubation time to obtain a detectable level of exoglycosidase activities which differs from enzyme to enzyme. Furthermore, assay linearity was also maintained longer for DBS than for fibroblasts. This effect was likely a result of the lower abundance of active enzymes found in DBS than in fibroblasts. At this point, DBS was not explored further, but future integration of DBS into new enzyme assays might require extended incubation times, perhaps 24 hours or more, to allow sufficient exoglycosidase activities to be detected. In the end, DBS remains a simpler sample type for collecting an enzyme source.

PART B: Determination of a universal buffer for exoglycosidase multiplexing

3.1 Introduction

In order to multiplex exoglycosidases within the same assay, the next step was to determine a universal buffer that would best represent the *in vivo* lysosomal environment while allowing exoglycosidases to function optimally *in vitro*. In the current 4MU-based fluorescence assay methods, each exoglycosidase uses a different set of buffer conditions with variable concentrations and pH (Table 3; p.29). Currently, there are three main buffer systems established for these lysosomal enzymes: citrate-phosphate, lactic acid-sodium hydroxide and sodium acetate-acetic acid. More specifically, β-Hexosaminidase, α-Mannosidase, β-Mannosidase, and α-Fucosidase assays generally uses a citrate-phosphate buffer, the β-Galactosidase assay uses a lactic acid-sodium hydroxide buffer and the α-Neuraminidase assay uses a sodium acetate-acetic acid buffer. In order to determine if one of these buffer species can be used as a universal buffer for all the exoglycosidases being studied, each enzyme assay was tested with every buffer condition.

3.2 Materials and Methods

The 4MU fluorescence assay procedures for the incubation and detection of individual exoglycosidases were carried out in the same manner as described in Part A, section 2.2.5. Exoglycosidase activities were determined individually and compared. After a universal buffer species was established, the concentration of the buffer components and the buffer pH were re-optimized to maximize activity levels for all exoglycosidases. Skin fibroblasts were used as the enzyme source throughout this analysis and the same procedures for enzyme collection were followed as described in Part A, section 2.2.3.

3.3 Results

3.3.1 Comparison of buffer species

Each exoglycosidase was examined under all six previously established buffer conditions and the resulting enzyme activities were expressed as a percentage of the activity determined under the native buffers for each individual enzyme (Figure 8; p.43). When the sodium acetate-acetic acid buffer was used, β-Hexosaminidase, β-Galactosidase, α-Mannosidase, and α-Fucosidase activities were compromised. The low activities observed meant that this buffer species would not make a good universal buffer. When the lactic acid-sodium hydroxide buffer was tested among the exoglycosidases, α-Mannosidase and α-Fucosidase activities were also significantly disrupted, which indicated that this was also not a good universal buffer candidate. Consequently, highest activity was observed for most exoglycosidases under the α-Fucosidase assay condition which used a citrate-phosphate buffer. In comparing all of the citrate-phosphate buffer conditions, it was noticed that a buffer with a lower citrate-phosphate concentration (0.03 - 0.05 M) and a lower pH (4.0 - 4.5) maximized measured exoglycosidase activities.

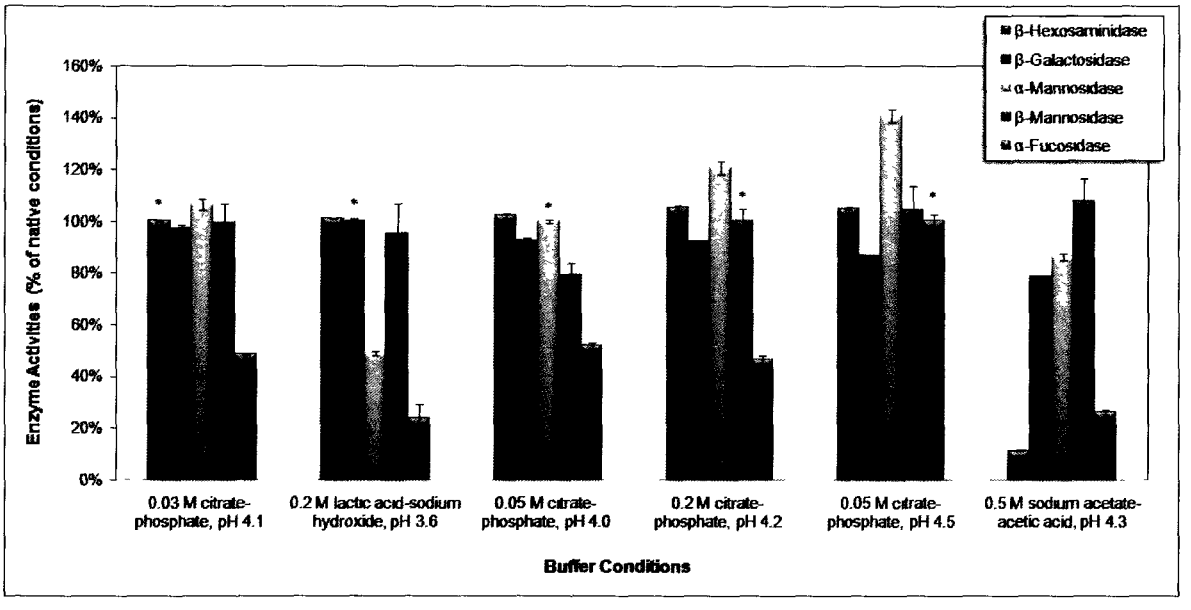


Figure 8. Exoglycosidase activities under different buffer conditions.

Activities for each lysosomal enzyme tested under the six buffer environments are expressed as a percentage of the native conditions, which are indicated by asterisks (*). Each enzyme was analyzed in duplicate for each of the different buffers. The error bars represent the mean $\pm$ SE.

3.3.2 Universal buffer optimization

A citrate-phosphate buffer was further evaluated to re-establish an optimal buffer concentration and pH. In one experiment, the citrate-phosphate pH was set at 4.2 (midpoint between the lowest and highest pH values in the previously established citrate-phosphate buffers) and citric acid was varied between 10 and 300 mM (Figure 9A; p.45). Most exoglycosidases showed higher activities at a buffer concentration below 50 mM. In a second experiment where the buffer concentration was set at 30 mM (lowest concentration used in the previously established citrate-phosphate buffers) and the pH was analyzed between 3.0 and 5.0 (Figure 9B), optimal enzyme activities were restricted to particular pH ranges. For β-Hexosaminidase and β-Galactosidase, this optimal range was larger and spanned from pH 3.7 - 4.4. For α-Mannosidase, β-Mannosidase and α-Fucosidase, this range was much narrower. These enzymes showed a sharp peak at pH 4.1, 4.2, 4.3, respectively. In the end, a 30 mM citrate-phosphate buffer with a pH at 4.3 was selected as the optimal universal buffer for further enzyme activity investigations.

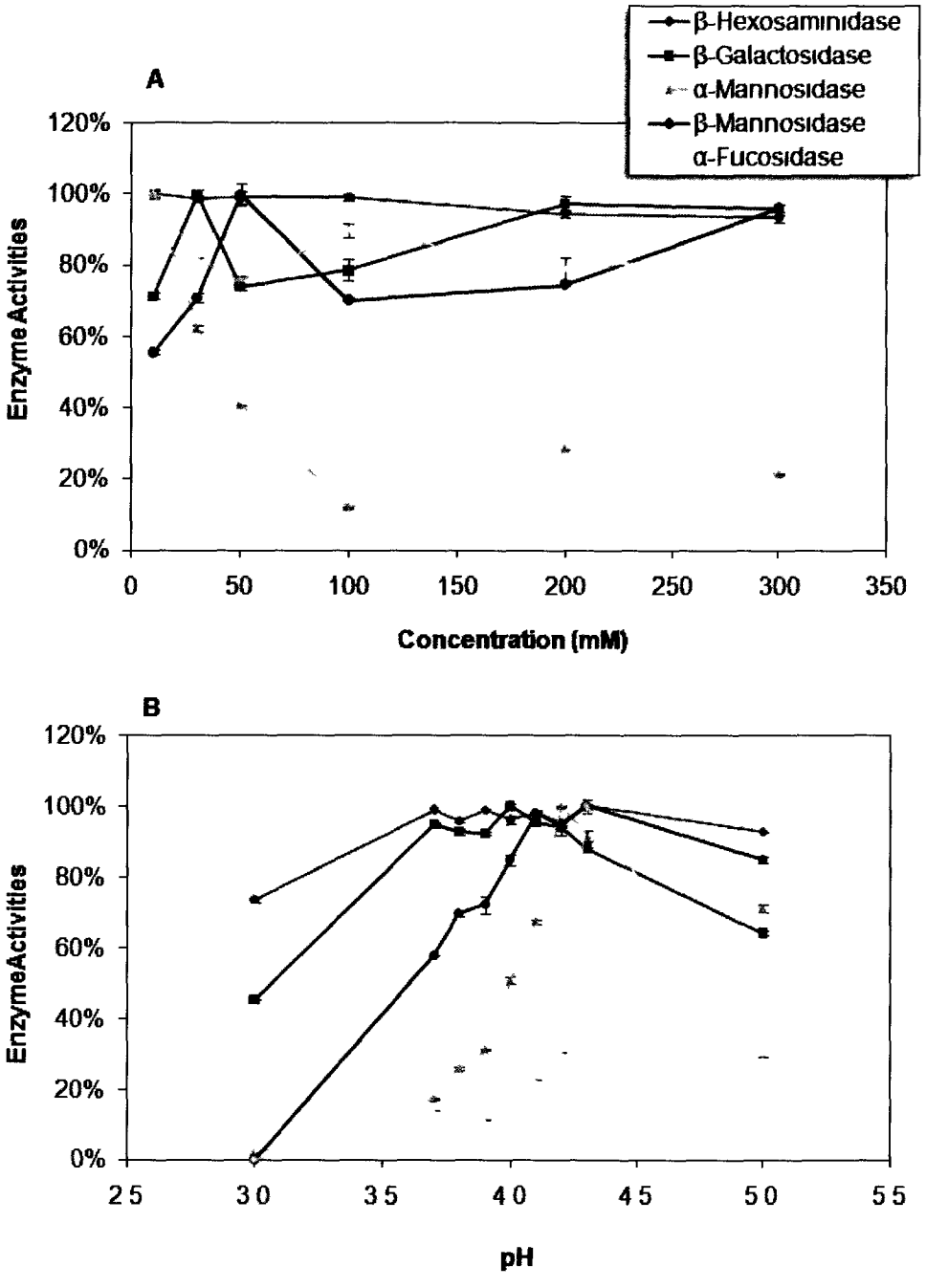


Figure 9. Optimization of a citrate-phosphate buffer.

(A) pH was set at 4.2 and the citrate-phosphate concentration was analyzed between 10 and 300 mM. (B) Citrate-phosphate concentration was set at 30 mM and pH was analyzed between 3.0 and 5.0. Each data point was collected in duplicate. The error bars represent the mean $\pm$ SE.

3.4 Discussion

Citrate-phosphate, lactic acid-sodium hydroxide and sodium acetate-acetic acid were the three distinct buffer species compared in this section to determine a universal buffer. It was very interesting to see that detectable activities could be obtained for all exoglycosidases under such a wide spectrum of buffer conditions. A citrate-phosphate buffer was successfully identified as the best buffer species to achieve highest activities for the group of exoglycosidases. Further optimization of this citrate-phosphate buffer by varying the pH and buffer concentrations revealed a specific optimal universal buffer with concentration at 30 mM at pH 4.3. In general, it was noted that the buffer pH was more critical than the buffer concentration for obtaining the highest enzyme activities.

In this analysis, each enzyme could only be assessed one at a time since all the assays involved measuring liberated 4MU. In order to generate a multiplex assay using this newly established universal buffer so multiple exoglycosidases could be detected under the same *in vitro* environment, different initial substrates producing unique enzymatic products had to be identified. Differentiation of these products in the same assay could be accomplished using MS/MS by detection of mass differences. MS/MS was explored in the next section as a new method for product identification which will allow multiplexing of the exoglycosidases.

PART C: Developing a quantitative MS/MS based exoglycosidase assay

4.1 Introduction

4.1.1 MS/MS technology

The electrospray ionization triple quadrupole tandem mass spectrometer (MS/MS) consists of five basic components: an ion source, a mass analyzer (MS1), a collision chamber, a second mass analyzer (MS2), and a detector (Figure 10; p.48) (Sansom 1999, Glish, Vachet 2003). When a sample is introduced into the MS/MS, a high voltage is applied to the electrospray probe causing the liquid sample containing the analyte to disperse into an aerosol of highly charged droplets. As nitrogen gas is presented, these charged droplets shrink in size by evaporation. This process, known as "electrospray ionization" (ESI), creates charged sample ions that are then released and pass into the analyzer of the MS/MS, which is under high vacuum. Ions of interest are selected according to their m/z by the first mass analyzer (MS1). These ions are generally termed "precursor or parent ions". In the collision chamber, molecules encounter an inert gas (e.g. argon) and fragment by collision induced dissociation (CID) to form smaller "product or daughter ions". Selection of the product fragments occurs in the second mass analyzer (MS2). Results of this detection are displayed in a graph known as a mass spectrum. In a mass spectrum, positions along the x-axis represent masses (m/z), whereas the height of a peak along the y-axis represents the quantity of ions, also known as "ion count" or "ion intensity". Monitoring the peak shape of a total ion chromatogram is important because it provides information on the relative quality of the injection and the analysis.

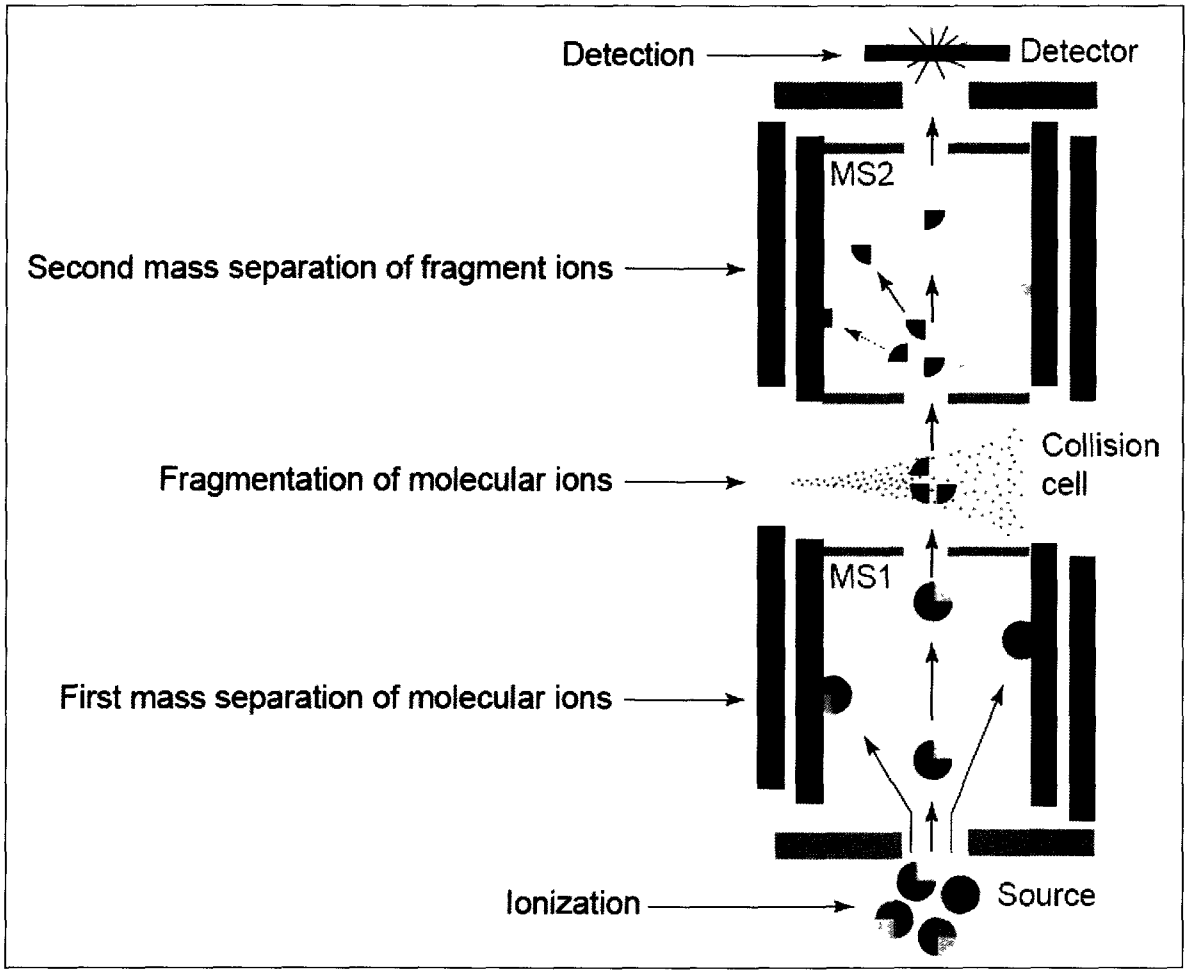


Figure 10. Schematics of a tandem mass spectrometer (MS/MS).

This instrumentation basically consists of an ion source, a mass analyzer (MS1), a collision chamber, a second mass analyzer (MS2), and a detector. Molecules are sorted by mass in the first spectrometer (MS1), then broken down into fragments by the collision cell and are sorted again by mass in the second spectrometer (MS2). The results are displayed by a computer as a mass spectrum. (Adapted from Sansom, 1999)

MS/MS can be used to investigate analytes of interest in many different ways. The use of computer algorithms allows for several types of MS/MS scan functions to be processed in a single analysis in a short period of time, generally two to three minutes. Two main operating modes are MS and MS/MS. In an MS operating mode, MS1 is used as the mass filter so that only precursor ions are monitored. In an MS/MS operating mode, both MS1 and MS2 are functional. Daughter ions are produced by CID when the collision gas is turned on which can be monitored by MS2. MS/MS scanning can be set-up in several different ways depending on the type of structure elucidation data required.

Three common MS/MS scan methods were used throughout this research (Figure 11; p.51). In the product (daughter) ion scan mode (Figure 11A), all fragments produced from a single precursor ion in MS1 are detected in MS2. In the precursor (parent) ion scan mode (Figure 11B), MS2 is set to detect for a certain daughter ion and all precursors producing the single product ion are detected in MS1. Both of these scanning techniques allow specific parent and daughter ions to be identified, and each is known as a "parent to daughter transition" (P/D transition). Multiple P/D transitions could be programmed to generate a scanning method known as "multiple reaction monitoring" (MRM) (Figure 11C), which can allow detection of several analytes from one sample injection. MRM provides greater analytical sensitivity and specificity since it has the ability to select particular analytes of interest based on both parent and daughter ion information. Accordingly, it is currently the most common technique used in newborn screening MS/MS protocols (Wilcken, Wiley 2008).

Another important enhancement to the MS/MS technology is the addition of a chromatography step to separate compounds of interest from a mixture prior to analysis by

MS/MS. The UPLC (Ultra Performance Liquid Chromatography) system together with MS/MS adds another level of sensitivity and specificity for analysis. UPLC-MS/MS was briefly investigated in this research for separation of monosaccharide isomers.

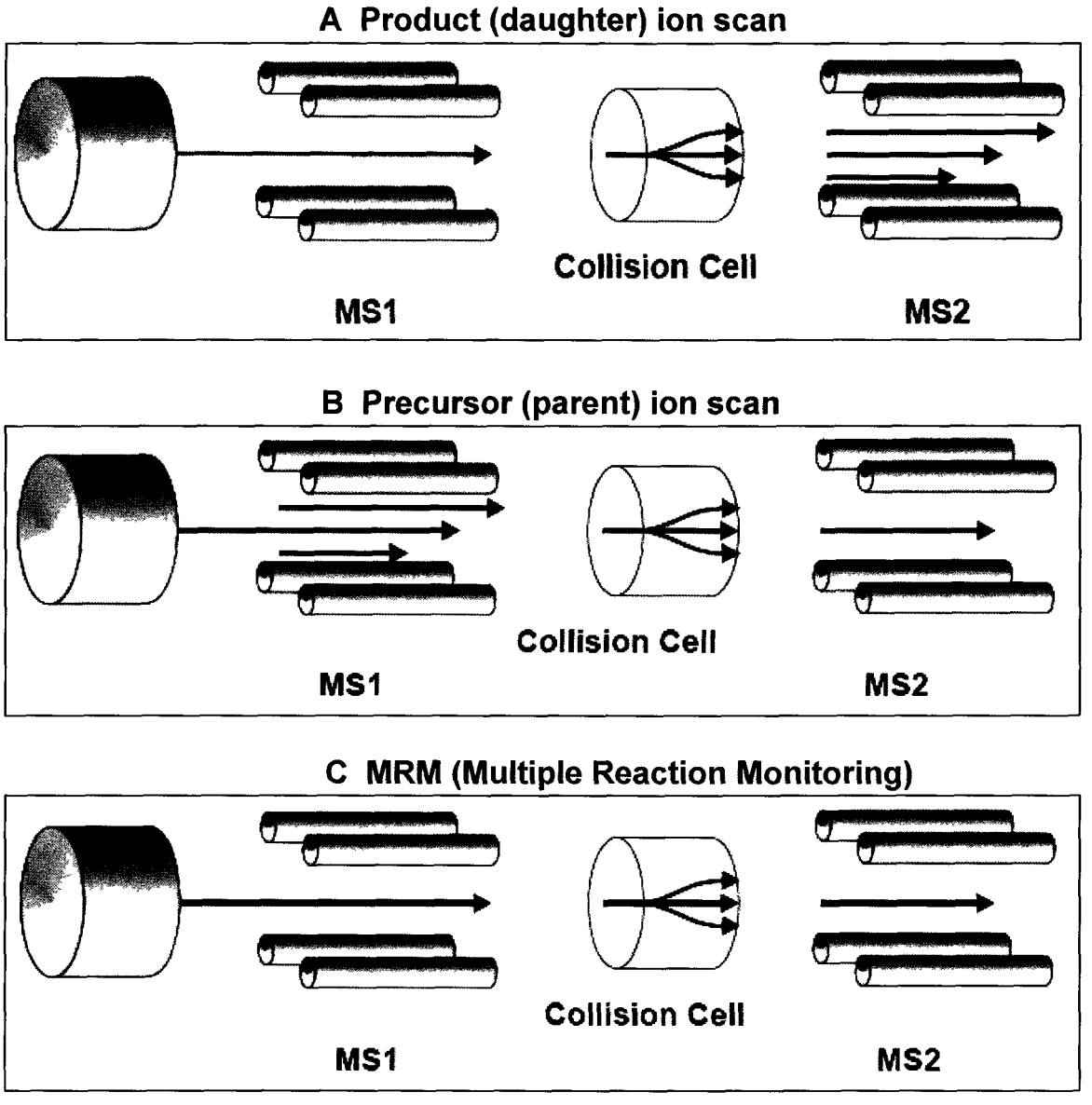


Figure 11. MS/MS scan modes.

A) Product (daughter) ion scan – all fragments produced from a single precursor are detected. B) Precursor (parent) scan – all precursors producing a single product are detected. C) MRM (Multiple Reaction Monitoring) – only specific parent to daughter transitions are detected. (Adapted from Waters, 2003)

4.1.2 Design of a MS/MS based multiplex assay for lysosomal exoglycosidases

As discussed earlier, Gelb et al. (Li et al. 2004b, Gelb et al. 2006) had successfully developed a MS/MS based multiplex method for diagnosing five treatable LSDs. The process begins with a unique set of artificial substrates and corresponding internal standards. After incubating with an enzyme source from DBS, the enzyme products are directly measured by MS/MS and enzyme activities can be calculated. The only downfall to this procedure is that the initial reactions require separate incubations before combining the end products and extra sample work-up steps are needed prior to MS/MS analysis. Both a liquid-liquid extraction step and a solid-phase chromatography step are needed to remove salts and other reagents that are incompatible with MS/MS.

In this research, an attempt was made to develop a new multiplex assay for the simultaneous diagnosis of Oligosaccharidoses using a similar MS/MS based product quantification and enzyme activity determination approach as in the Gelb et al. method. Initially, a new multiplexing strategy using carbohydrate derivatization was proposed, which was intended to simplify the previous method so individual reactions could be processed and analyzed together in a single tube.

First, disaccharides containing the natural glycosidic linkages recognized by the specific exoglycosidases were chosen as the new initial substrates. In theory, enzymatic cleavage of these disaccharides should produce unique monosaccharide products that could be differentiable by MS/MS based on mass differences. A quick comparison of the probable degraded monosaccharide structures revealed two initial conflicts for MS/MS analysis (Figure 12; p.53). One is that the monosaccharides on their own are not readily ionizable by ESI since they do not contain functional groups that would allow them to gain

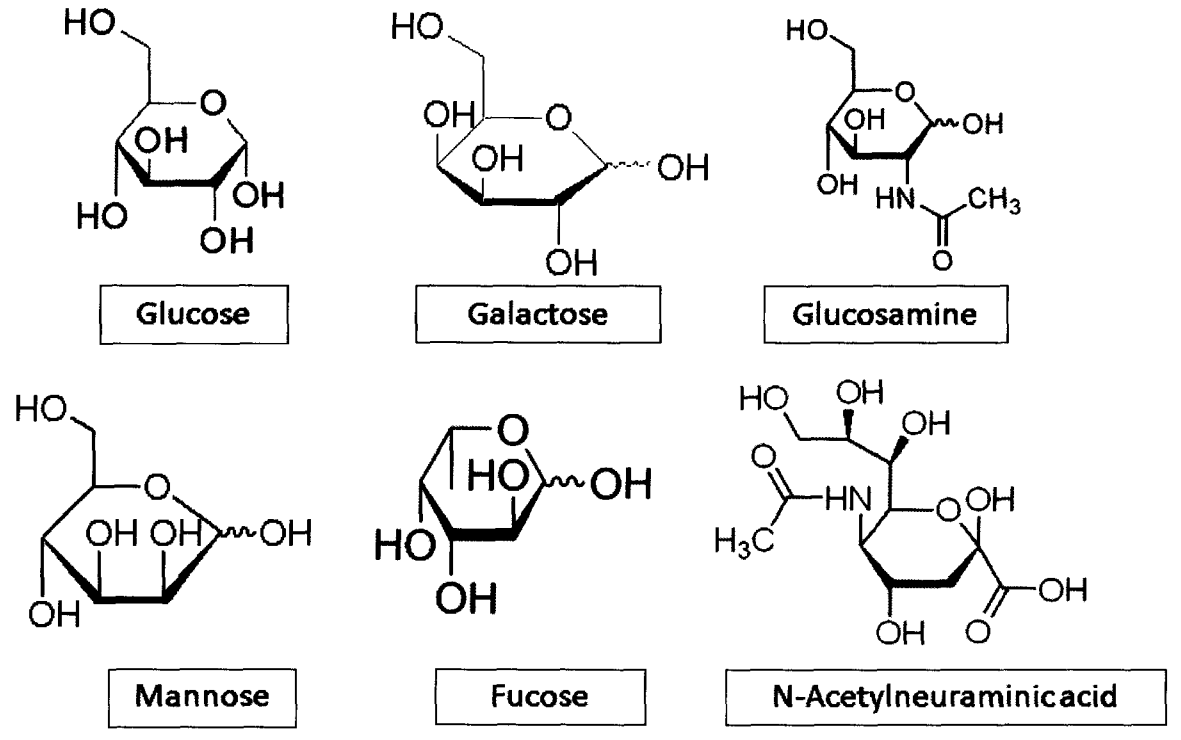


Figure 12. Target monosaccharide degradation products.

Glucose, galactose, glucosamine, mannose, fucose and N-acetylneuraminic acid are potential monosaccharide products that result from N-linked oligosaccharide catabolism. Monitoring these carbohydrate structures by MS/MS after enzymatic reactions within a single reaction tube could allow simultaneous detection of Oligosaccharidoses. Of note, glucose, galactose and mannose possess the same molecular weight, which would be a challenge to differentiate individually by MS/MS.

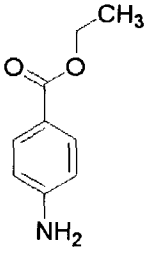
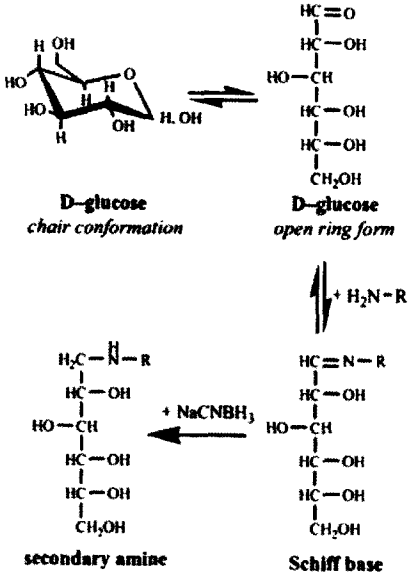
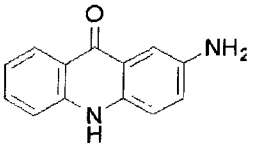
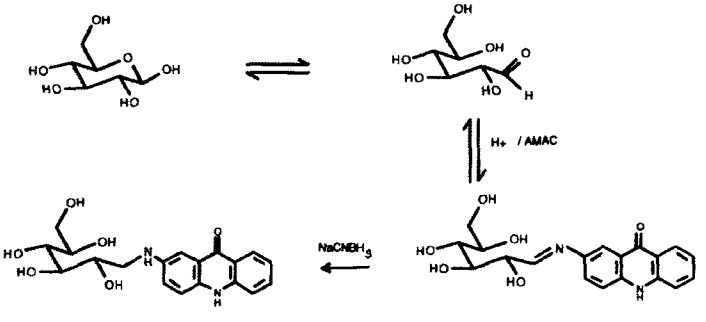
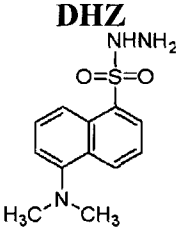
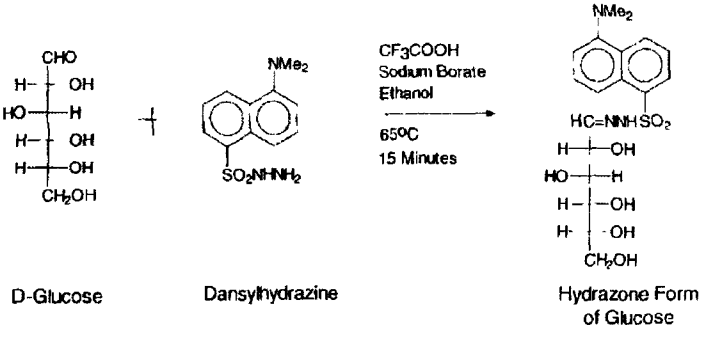
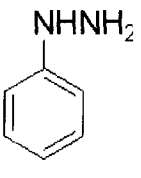
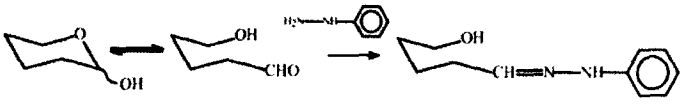
or lose a charge. As a result, MS/MS will not be able to detect these molecules. The second problem is that many of these monosaccharides are isomers. Even though the compounds differed in structure, many possess the same molecular weight (e.g. glucose, galactose and mannose) and this renders differentiation by MS/MS difficult when the monosaccharides are presented in the same reaction tube.

To resolve these issues, "derivatization" was introduced. This is a sample preparation technique where another compound is chemically added to the analyte of interest to allow the final product to contain specific new properties to facilitate detection. For this research purpose, the goal of derivatization was to create charged carbohydrate structures, so they could be detected by MS/MS. Additionally, if derivatization agents of differing mass could be used and be equally detectable by MS/MS, it would resolve the dilemma of differentiating isobaric monosaccharide isomers in the same reaction.

An earlier study conducted by Ramsay et al. (Ramsay et al. 2005), demonstrated a successful assay for the diagnosis of several Oligosaccharidoses by the use of 1-phenyl-3-methyl-5-pyrazolone (PMP) in derivatization of urinary oligosaccharides prior to analysis by MS/MS. In this research study, urine was not the sample type of interest, but the use of PMP as a derivative candidate for assisting oligosaccharide detection by MS/MS was a new technique that deserved further investigations.

Previous literature also described four general types of derivatization chemistry for carbohydrates which included reductive amination, amine coupling, hydrazone formation and condensation with a methylene group (Suzuki, Kakehi & Honda 1996, Honda, Suzuki & Taga 2003, Lamari, Kuhn & Karamanos 2003, Lattova et al. 2005). Several reagents were selected from each of these reaction types and derivatization was tested on various

carbohydrates to determine suitable candidates for assisting MS/MS analysis of oligosaccharides (Figure 13; p.56-57). 4-Aminobenzoic acid ethyl ester (ABEE) and 2-aminoacridone (AMAC) required reductive amination reactions, where a Schiff base is formed between the amino group in the amine reagent and the reducing end of the reducing carbohydrate. Phenylhydrazine (PNH), dansylhydrazine (DHZ), Girard's T reagent and 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole (DAABD-MHz) involved another type of reaction, which formed a hydrazone by nucleophilic attack of the hydrazine and the hemiacetal carbon of the carbohydrate. Carbohydrate derivatization with DAABD-MHz had never been investigated before, but reports have shown that this compound could generate unique fragmentation patterns that could be easily detected by MS/MS (Santa et al. 2008). PMP derivatization used a condensation method where the reaction occurs between an active methylene group in the pyrazolone ring and the aldehyde group of the carbohydrate.

	Derivative	Reaction
Reductive Amination	**ABEE** 	 **D-glucose** *chair conformation* **D-glucose** *open ring form* + H_2N-R + $NaCNBH_3$ **secondary amine** **Schiff base**
	AMAC 	 H^+ / AMAC $NaCNBH_3$
Hydrazone Formation	**DHZ** 	 CF_3COOH Sodium Borate Ethanol 65°C 15 Minutes D-Glucose Dansylhydrazine Hydrazone Form of Glucose
	PNH 	

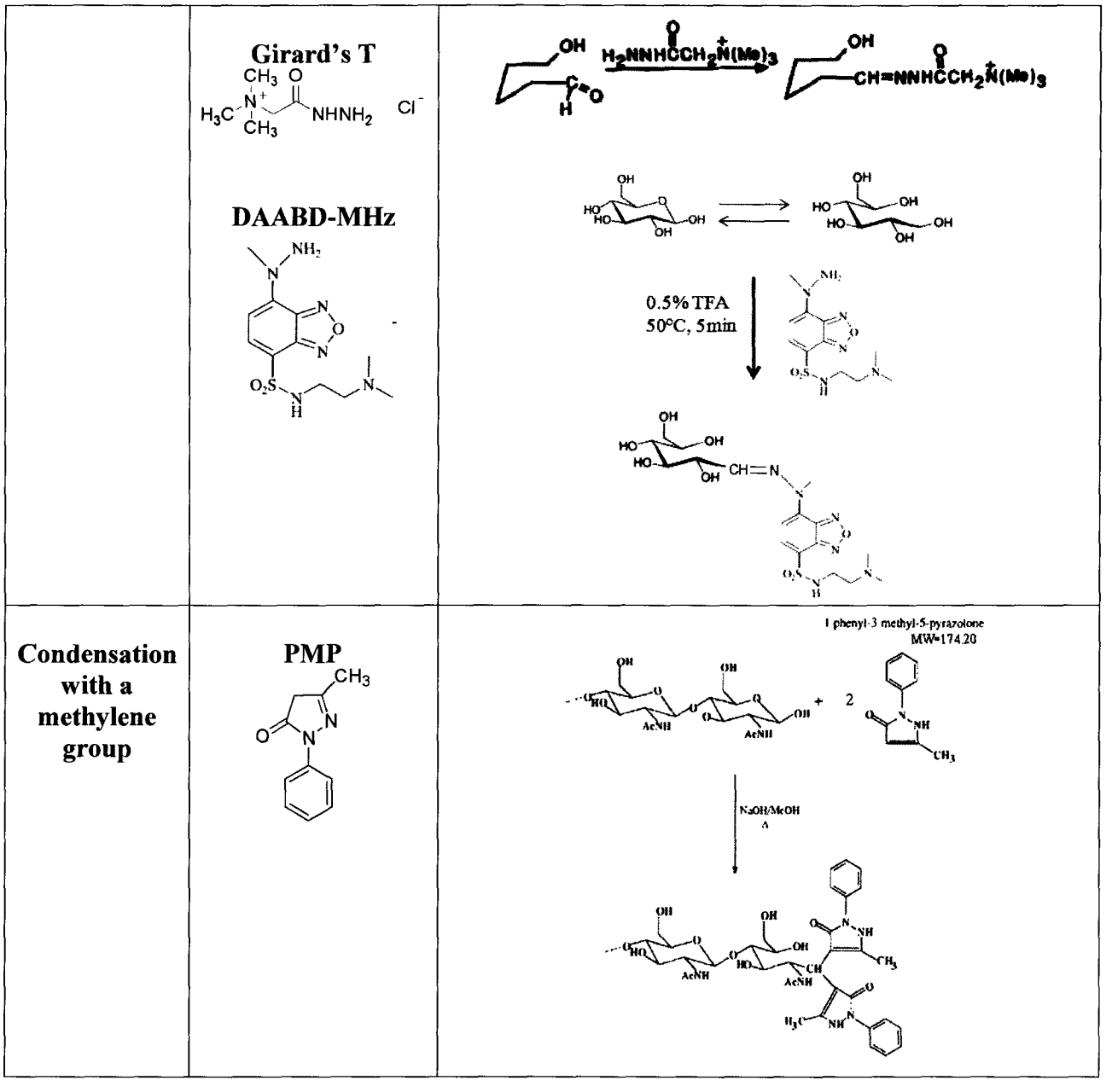


Figure 13. Common carbohydrate derivatization reactions.

Molecular structures of common reagents described in previous literature for analysis of carbohydrates by MS/MS. Addition of these compounds to carbohydrates at the reducing end will facilitate ESI and increase MS/MS analytical sensitivity. Reaction schemes of selected carbohydrate derivatization methods tested in this research are illustrated.

In the conventional lysosomal enzyme assay, enzyme activity was determined by monitoring the appearance of a unique product after a substrate of the enzyme was exposed to an enzyme source. Typically end-point assays were used. To integrate a derivatization step into a new assay which used a disaccharide as the initial substrate for assisting MS/MS detection of particular exoglycosidase products, there were actually two ways that this could be accomplished, either before or after the enzymatic reaction. In a pre-reaction derivatization method, addition of a reagent occurs at the reducing end of the starting disaccharide substrate. Therefore, after the specific enzyme cleaves the disaccharide glycosidic linkage, one monosaccharide product should theoretically contain the derivative. In contrast, post-reaction derivatization would result in the two monosaccharide products that would gain a derivative compound since each monosaccharide has a free reducing end for the derivative to attach. Monitoring one unique monosaccharide product by MS/MS would be sufficient to demonstrate its activity. In theory, multiplexing could be accomplished by careful selection of initial oligosaccharide substrates targeting each exoglycosidase of interest and by combining both pre- and post-reaction derivatization methods using two or more derivatives to differentiate isomeric monosaccharide products. A hypothetical reaction scheme for the multiplexing of α-Neuraminidase, β-Galactosidase, β-Hexosaminidase, α-Mannosidase, β-Mannosidase and α-Fucosidase is illustrated in Figure 14 (p.59). Carrying out a pre-reaction derivatization step with PMP on particular substrates and a post-reaction derivatization step with DHZ on the remaining products would allow a collection of derivatized monosaccharides that could be analyzed in the same reaction tube. Programming the MS/MS to monitor one specific product for each exoglycosidase reaction would allow the simultaneous diagnosis of the Oligosaccahridoses.

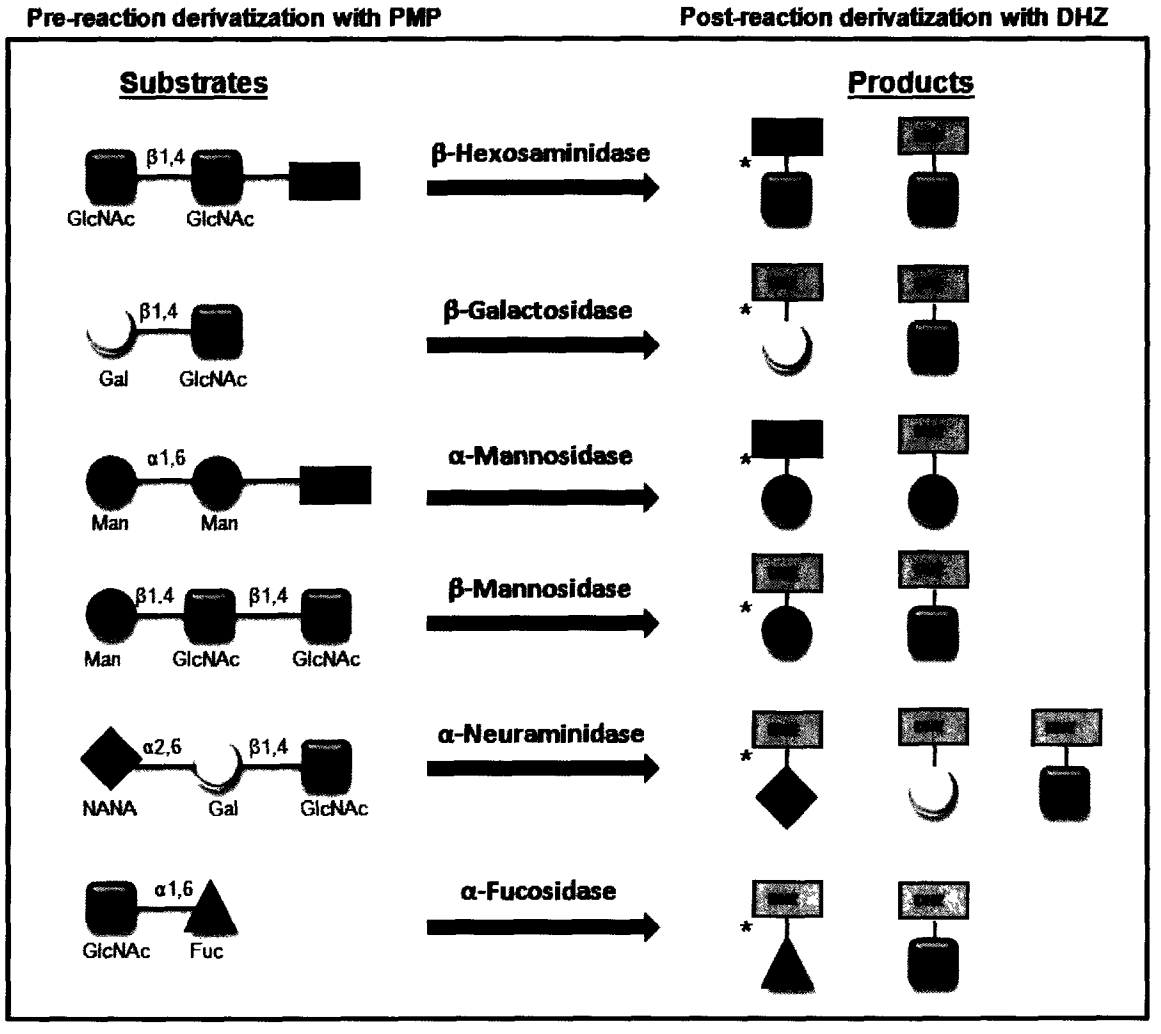


Figure 14. Potential reaction scheme of an exoglycosidase multiplex assay.

Pre-reaction derivatization of select initial oligosaccharide substrates would allow specific monosaccharide products to contain a derivative, such as 1-phenyl-3-methyl-5-pyrazolone (PMP). Performing another post-reaction derivatization step with an alternative reagent, such as dansylhydrazine (DHZ), would allow the monosaccharide end products to become labeled. Selection of unique monosaccharide products as indicated by asterisks (*) would allow isomeric monosaccharide differentiation by MS/MS based on molecular mass differences.

Initially, each exoglycosidase was individually analyzed to optimize assay conditions and MS/MS detection parameters before multiplexing of the assays were investigated. Carbohydrate derivatization methods were explored first since natural oligosaccharide substrates were not good analytes for MS/MS detection. Internal standard quantification techniques were also studied for monitoring exoglycosidase activities through quantifying appropriate derivatized monosaccharide products by MS/MS.

4.2 Materials and Methods

4.2.1 Derivatization with PMP

20 µl (20 µg) of a 1 mg/ml stock of sugar in H_2O was added into a new Eppendorf tube and dried *in vacuo*. 30 µl of 0.3 M NaOH and 30 µl of 0.5 M PMP in methanol solution were added. The tube was heated for 30 minutes at 70°C in a heating block. Samples were cooled to room temperature and 30 µl of 0.3M HCl was added. Contents were dried *in vacuo* and 2 x 500 µl of CH_3CN was used for PMP-sugar extraction. The two CH_3CN supernatants were collected together and dried *in vacuo*. Samples were stored at -20°C or reconstituted in [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] for analysis by MS/MS.

4.2.2 Derivatization with DHZ

20 µl (20 µg) of a 1 mg/ml stock sugar solution in H_2O was added into an Eppendorf tube and dried *in vacuo*. 20 µl of 4 mg/ml DHZ in CH_3CN and 20 µl of 0.2% (v/v) trichloroacetic acid (TCA) were added. Derivatization was carried out at 40°C for 75 minutes. Samples were dried for 30 minutes and either stored at -20°C or reconstituted in [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] for analysis by MS/MS.

4.2.3 Pre-reaction derivatization with PMP

Disaccharide substrates at 1 mM were prepared in H_2O. 50 µl was dried *in vacuo* and PMP derivatization was carried out following the procedures from 4.2.1. The derivatized substrate was reconstituted in the 30 mM citrate-phosphate universal buffer at pH 4.3 and appropriate amounts of enzyme were added to initiate the reaction. Reactions were boiled for 5 minutes to stop the enzymatic reaction. Content was dried *in vacuo* for 2.5 hours. 500 µl of CH_3CN was used to collect the carbohydrate products. Samples were centrifuged at 13.2 rpm for 2 minutes. Supernatant was dried and reconstituted in [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] for analysis by MS/MS.

4.2.4 Post-reaction derivatization with PMP or DHZ

Enzyme reaction containing appropriate amounts of enzyme, universal buffer (30 mM citrate-phosphate, pH 4.3) and disaccharide substrate were incubated for the necessary amount of time. Reactions were boiled for 5 minutes to stop the enzymatic reaction. Content was dried *in vacuo* for 2.5 hours. 500 µl of CH_3CN was used to extract the carbohydrate products. Samples underwent a quick spin at 13,200 rpm for 2 minutes. Supernatant was dried and products were derivatized with PMP or DHZ following the optimized procedures in 4.2.1 or 4.2.2. Dried samples were reconstituted [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] for analysis by MS/MS.

4.2.5 Tandem mass spectrometry

Mass spectrometry analysis of carbohydrates was performed using the Quattro Premier XE Mass Spectrometer with an ESI source and an ACQUITY UPLC sampler system (Waters, Milford, MA, USA). For analyzing samples in the precursor (MS) or

product (MS/MS) ion scan modes, samples were injected by direct infusion into the ESI source at a flow rate of 10 µl/min. For analyzing samples in the MRM scan mode, 10 µl samples were injected into the ESI source by the autosampler using a carrying solvent of [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] at a flow rate of 0.1 ml/min. All derivatized carbohydrates were analyzed in the positive ion mode.

4.3 Results

4.3.1 MS/MS and mobile phase optimization

For the analysis of each new compound, several parameters on the MS/MS must be optimized to obtain maximum peak signals, including the cone voltage (CV) and collision energy (CE). Data collected permit P/D transitions to be identified for subsequent preparation of the more sensitive MRM scanning technique. In Figure 15 (p.64), an example for the optimization of these parameters to identify one pair of P/D transition is illustrated for PMP-glucose (m/z=511). Optimal values were set when the highest peak intensity was determined. The CV is the energy applied to the sampling cone to allow ions produced by ESI to accelerate through to MS1 of the MS/MS, which is held under high vacuum. Parent ions were optimized using this parameter by adjusting CV at increasing increments of 10 and optimal CV was found to be 40 V (Figure 15A). Of note, as the CV is increased further, "in-source fragmentation" can occur, where weaker bonds such as C-N and C-O bonds are spontaneously broken. Fragments that appear in MS1 are not daughter ions and are not necessarily informative. Therefore, optimizing CV needs to be accomplished carefully. The CE is the potential applied to control the energy of the collisions between particular parent ions and the argon atoms in the collision chamber to produce daughter ions which are detected by MS2. Daughter ions were optimized using

this parameter by starting at a CE of 20 and increases at increments of 2 were analyzed. Optimal CE was determined to be 28 eV (Figure 15B). From the MS/MS scans, a unique daughter fragment at 175 m/z was observed, therefore a P/D transition was established at 511>175. Other derivatized sugars were tested in the same manner and several specific P/D transitions were identified (Table 5; p.65). These sugars were analyzed separately, but eventually these would be the potential substrates and reaction products that would be monitored in the new exoglycosidase assays.

Another important component of mass spectrometry is the mobile phase which acts as the carrying solvent to facilitate ESI. Furthermore, selecting an appropriate mobile phase also assists liquid chromatography separation techniques. Typical solvents were prepared by mixing water with volatile organic compounds such as acetonitrile (CH_3CN) or methanol (MeOH). Composition is sometimes compromised by what gives the best ionization and what gives the best liquid chromatography separation. Therefore, additives are often added to assist both of these situations. A quick comparison of using CH_3CN versus MeOH as the organic portion in the mobile phase revealed that CH_3CN gave higher peak intensities for most derivatized carbohydrates (data not shown). Different mobile phases with varying compositions of CH_3CN and H_2O were further assessed. Additives such as formic acid (CH_2O_2) and ammonium acetate (NH_4OAc) were also tested in these mobile phases to look for improvements in peak resolution. An [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] was found to be the optimal mobile solvent for analyzing these derivatized sugars as shown in Figure 16 (p.66) analyzing PMP-GlcNAc(β1,2)Man (m/z=714). This mobile phase and the optimized MS/MS parameters were used throughout the rest of this research.

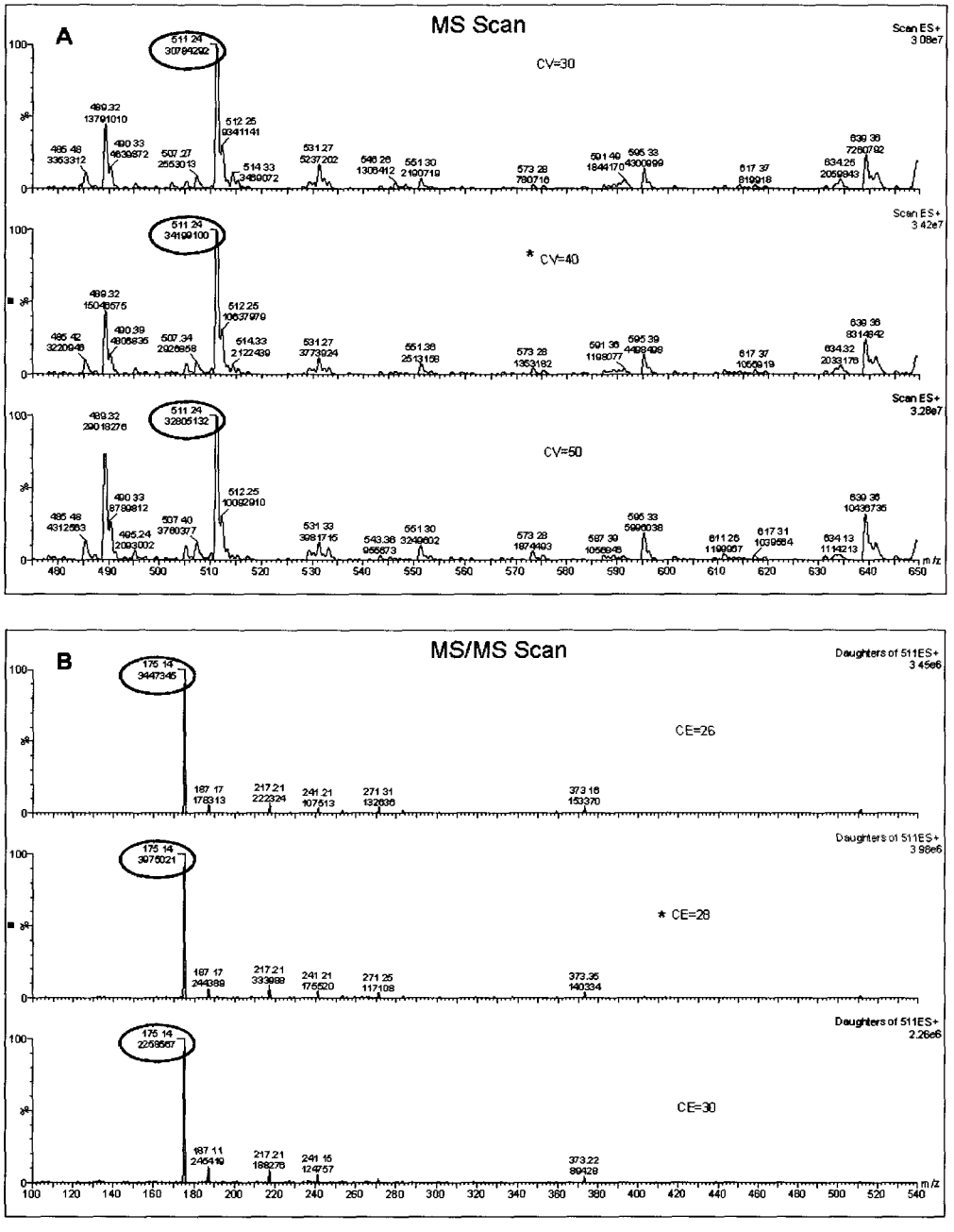


Figure 15. Optimization of MS/MS parameters for PMP-mannose.

Each spectrum displays peaks at varying m/z which are plotted against the % intensity. The upper value above each peak represents the m/z and the lower value represents the ion counts. A) MS scans show that the optimal CV for PMP-mannose is at 40 V. B) MS/MS scans show that the optimal CE for PMP-mannose is at 28 eV. Optimal values are highlighted by asterisks (*).

Table 5. Optimal MS/MS parameters for various derivatized sugars.

Monosaccharide + Derivative	Cone Voltage (V)	Collision Energy (eV)	Parent Ion (m/z)	Daughter Ion (m/z)
PMP-Glu	40	28	511.56	175.20
PMP-Gal	40	27	511.56	175.20
PMP-GlcNAc	40	30	552.61	175.20
PMP-GalNAc	40	30	552.61	175.20
PMP-Man	40	30	511.56	175.20
PMP-$^{13}C_6$-Man	40	30	517.56	175.20
ABEE-Glu	26	24	330.35	166.19
ABEE-Gal	26	24	330.35	166.19
ABEE-GlcNAc	26	24	371.40	166.19
ABEE-GalNAc	28	30	371.40	166.19
ABEE-Man	26	24	330.35	166.19
PNH-Glu	10	12	271.30	109.14
PNH-Gal	14	12	271.30	109.14
PNH-GlcNAc	16	10	312.35	109.14
PNH-GalNAc	12	12	312.35	109.14
PNH-Man	10	12	271.30	109.14
AMAC-Gal	34	25	373.39	211.23
AMAC-GlcNAc	34	26	414.44	211.23
AMAC-Man	30	15	373.39	211.23
DAABD-MHz-Gal	30	25	478.16	316.00
DAABD-MHz-GlcNAc	30	25	519.21	316.00
DAABD-MHz-Man	30	25	478.16	316.00
DAABD- MHz-$^{13}C_6$-Man	30	25	484.16	316.00
Girard's T-Man	40	-	330.80	168.64
Girard's T-$^{13}C_6$-Man	40	-	336.80	168.64
DHZ-Glu	27	22	428.50	266.34
DHZ-GlcNAc	27	22	469.55	266.34
DHZ-Man	27	22	428.50	266.34
DHZ-$^{13}C_6$-Man	27	22	434.50	266.34

Disaccharide + Derivative	Cone Voltage (V)	Collision Energy (eV)	Parent Ion (m/z)	Daughter Ion (m/z)
PMP-Gal (β1,4) GlcNAc	40	30	714.75	175.20
PMP-GlcNAc (β1,2) Man	40	30	714.75	175.20
PMP-Man (α1,3) Man	36	36	673.70	175.20
PNH-Gal (β1,4) GlcNAc	10	-	474.49	109.14
PNH-Man (α1,3) Man	14	-	433.44	109.14
AMAC-Gal(β1,4) GlcNAc	34	15	576.58	211.23
AMAC-Man (α1,3) Man	34	20	535.53	211.23
DHZ-Gal (β1,4) GlcNAc	27	22	631.69	266.34
DHZ-Man (α1,3) Man	27	22	612.64	266.34
DHZ-GlcNAc (β1,2) Man	27	22	631.69	266.34

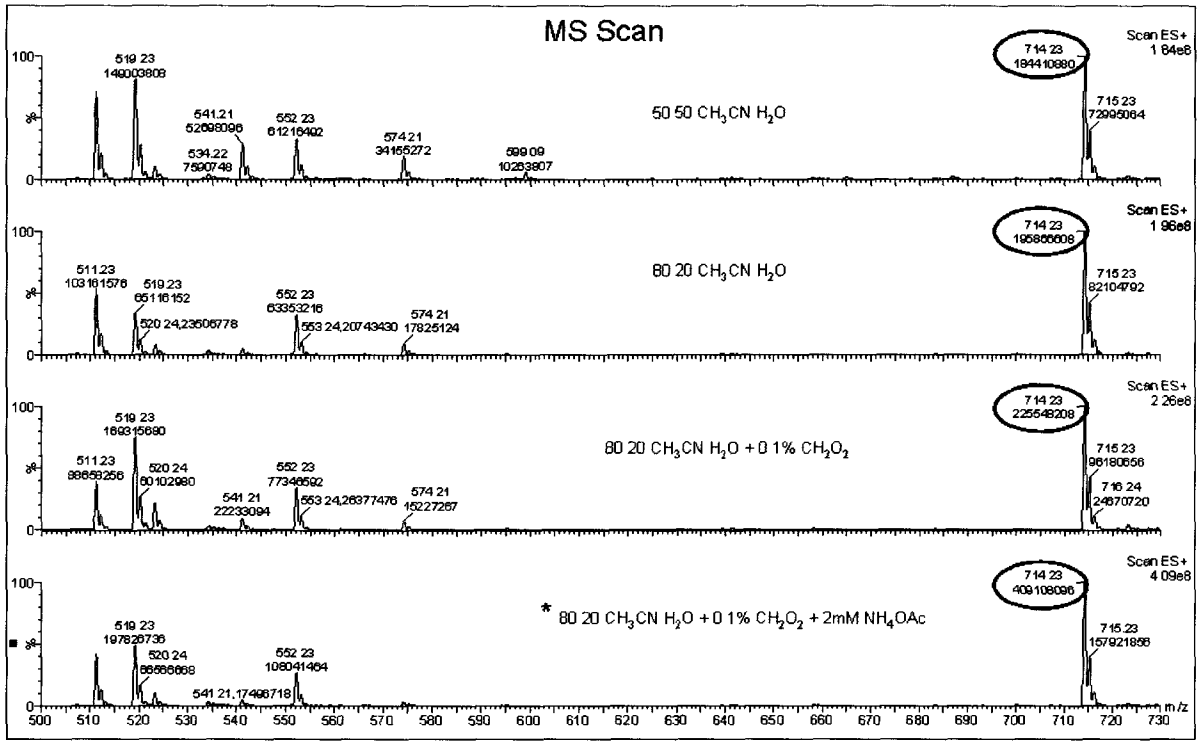


Figure 16. Optimization of mobile phase for PMP-GlcNAc(β1,2)Man.

Two different compositions of CH_3CN:H_2O and the effect of the formic acid (CH_2O_2) and ammonium acetate (NH_4OAc) additives were compared to determine the optimal mobile phase for PMP-GlcNAc(β1,2)Man. Optimized mobile phase is highlighted by an asterisk (*).

4.3.2 Comparison of derivatives and optimization of PMP and DHZ methods

A closer look at the signal intensities obtained from the various derivatization methods revealed that some reagents were more efficient than others for assisting the detection of carbohydrates by MS/MS. Using mannose as the test analyte and starting with the same amount in each analysis, different reagents were compared (Figure 17; p.68). Interestingly, DHZ gave the highest mannose signal. PMP gave the second highest signal, and the remaining reagents displayed a mannose peak with minimal intensities that were almost undetectable by MS/MS. DHZ and PMP were further examined as potential candidates for the reaction scheme described in Figure 14 (p.59).

The derivatization methods for both PMP and DHZ were further optimized. The PMP derivatization protocol was consistent throughout literature, but the final clean-up step which required multiple extractions (3x) with chloroform ($CHCl_3$) to remove excess PMP was found to be cumbersome. As a result, other organic solvents were investigated and acetonitrile (CH_3CN) was found to give better PMP-mannose recovery with only one extraction step (Figure 18; p.69). Alternatively, more work needed to be done to optimize the DHZ derivatization method because literature describing the reaction conditions for carbohydrates was old and inconsistent. Several parameters such as the reaction time, temperature, DHZ and TCA concentrations were re-evaluated (Figure 19; 70). Both the old and new PMP and DHZ derivatization methods are summarized in Table 6 (p.71). Of note, the total sample preparation times in the new PMP and DHZ methods were significantly shortened. Interestingly, detection of PMP-mannose and DHZ-mannose increased two folds after improvements were made to the previous published procedures (Figure 20; p.71). Overall, DHZ consistently gave a higher signal than PMP during MS/MS analysis.

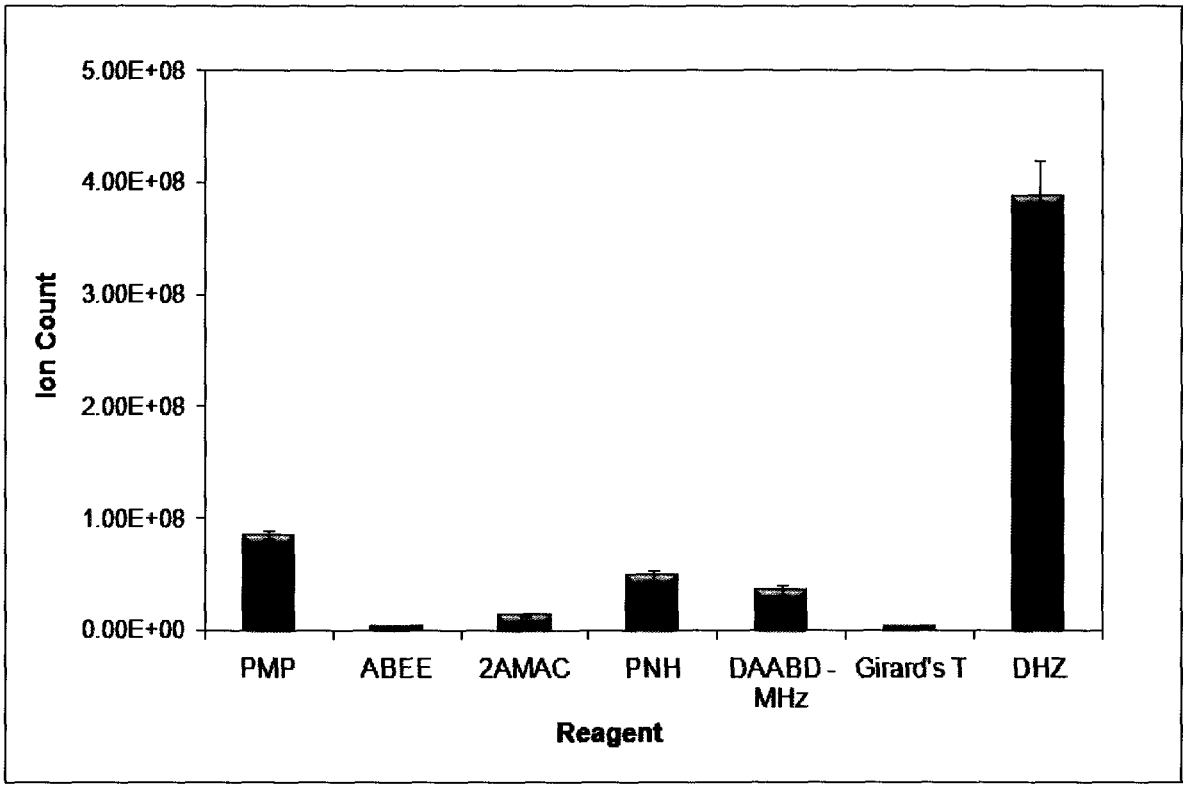


Figure 17. MS/MS comparison of mannose and different reagents.

Each carbohydrate derivatization method was performed in triplicate. The average intensities of the precursor ions are represented in ion counts. The error bars represent the mean ± SE. Refer to Figure 13 (p.56-57) for details of chemical reactions.

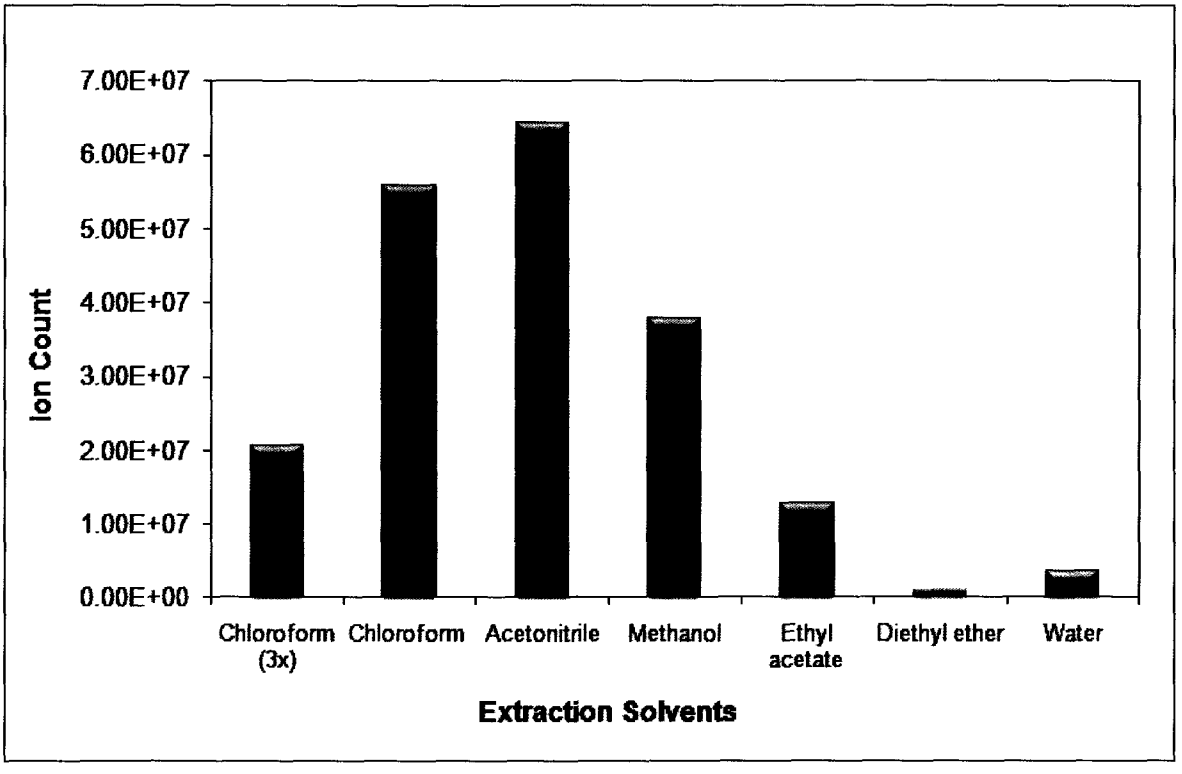


Figure 18. Optimization of the PMP derivatization method.

PMP-mannose was used as the test analyte. In the original method, the final step requires 3 successive extractions with chloroform to remove excess PMP. This step was modified to a one step extraction and various organic solvents were examined. PMP-mannose recovery was analyzed by MS/MS and the ion counts are displayed. There was only one opportunity to examine this step.

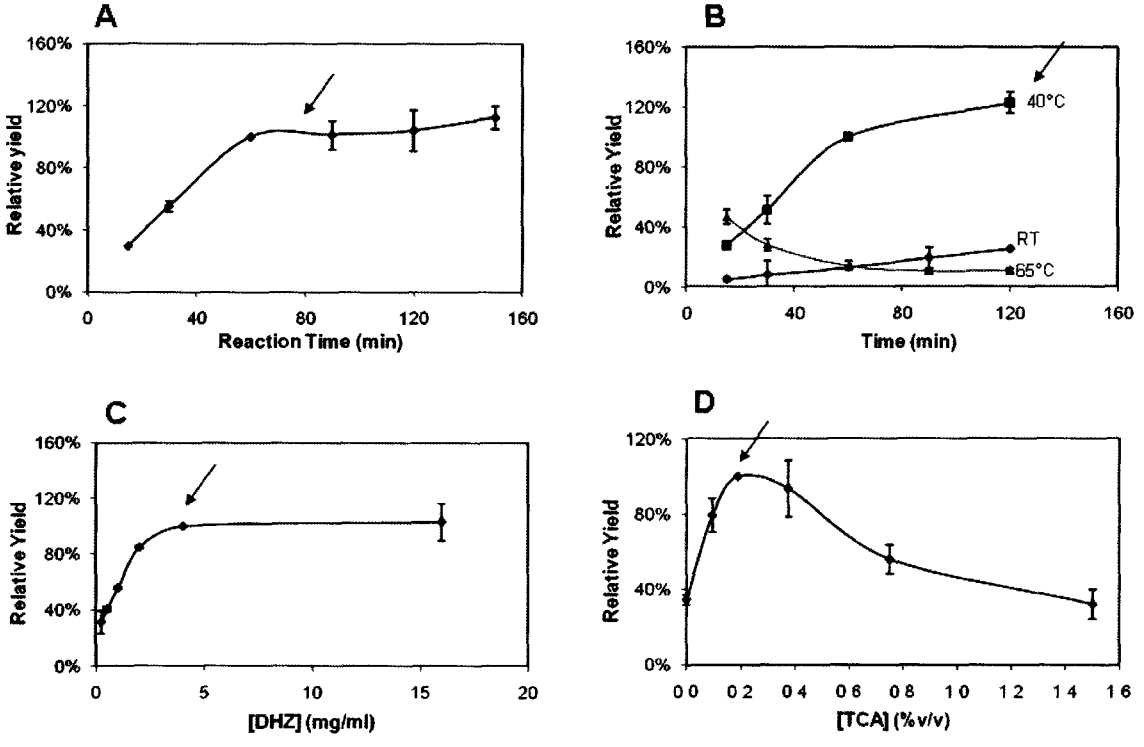


Figure 19. Optimization of the DHZ derivatization method.

PMP-mannose was the test analyte. The reaction time (A), reaction temperature (B), DHZ (C) and TCA (D) concentrations were optimized. PMP-mannose recovery was analyzed by MS/MS and the ion counts are represented as a % yield of the optimal value determined (indicated by red arrow). Samples were tested in duplicate. The error bars represent the mean $\pm$ SE.

Table 6. PMP and DHZ carbohydrate derivatization before and after optimization.

PMP Derivatization		DHZ Derivatization	
Published Procedure	**After Optimization**	**Published Procedure**	**After Optimization**
10 ng sugar ↓ Dry 30 min ↓ 30µl 0.5M PMP + 30µl 0.3M NaOH ↓ Heat at 70°C, 30 min ↓ 30µl 0.3M HCl ↓ Extract 3x with 500µl $CHCl_3$ ↓ Dry aqueous layer **Total preparation time ~ 3h**	10 ng sugar ↓ Dry 30 min ↓ 30µl 0.5M PMP + 30µl 0.3M NaOH ↓ Heat at 70°C, 30 min ↓ 30µl 0.3M HCl ↓ Extract 1x with 500µl CH_3CN ↓ Dry aqueous layer **Total preparation time ~ 2h**	10 ng sugar ↓ Dry 30 min ↓ 50µl 5% DHZ + 10µl 10% TCA ↓ Heat at 65°C, 20 min ↓ Dry aqueous layer **Total preparation time ~ 1.5h**	10 ng sugar ↓ Dry 30 min ↓ 20µl 4mM DHZ + 20µl 0.2% TCA ↓ Heat at 40°C, 75 min ↓ Dry aqueous layer **Total preparation time ~ 2h**

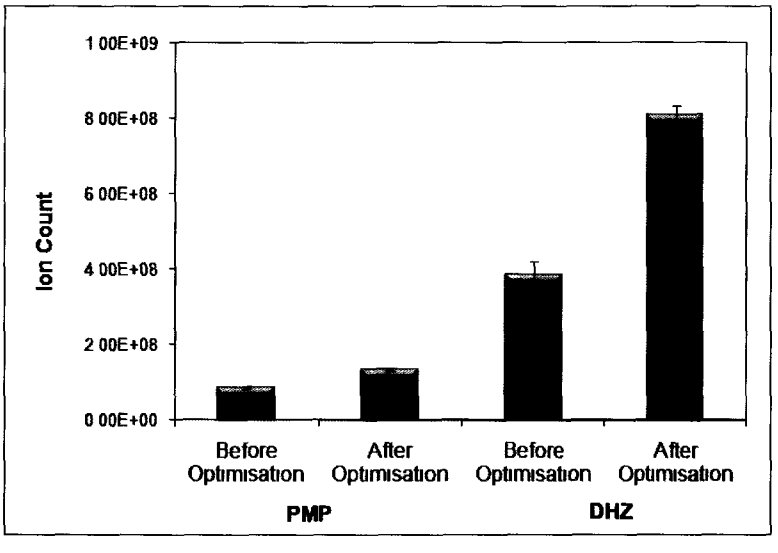


Figure 20. Comparison of PMP-mannose and DHZ-mannose detection by MS/MS. PMP and DHZ derivatization methods were optimized. Ion counts of the precursor ions are shown and the error bars represent the mean ± SE.

4.3.3 Generating a mannose standard curve with $^{13}C_6$-mannose as internal standard for enzymatic product quantification

Before attempting to run an enzymatic assay to measure enzyme activity by using a particular disaccharide as the starting substrate and incorporating a derivatization step to identify unique monosaccharide products, an internal standard was first chosen and tested in parallel with a target monosaccharide to devise a product quantification method. The internal standard should be chemically identical to the enzymatic product, but distinguishable by MS/MS through a mass difference.

Mannose and a commercially available isotope, $^{13}C_6$-mannose, were arbitrarily selected for investigation. These compounds were derivatized before analysis was done by MS/MS. The MRM scanning technique was used so that two P/D transitions could be programmed to specifically detect mannose and $^{13}C_6$-mannose at the same time. To collect MRM data, a "flow injection analysis" needed to be set-up between the MS/MS and the AQUITY sampler without chromatography. This required a constant solvent flow [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] through the MS/MS system and samples were automatically injected by the AQUITY sampler into this flowing system. Sample extracts of 10 µl were injected into the flowing stream which was operating at 100 µl/min. A chromatogram was initially generated (Figure 21A; p.74) which represents the time that the sample reaches the detector and the length of time scans were collected in this region. Integration over this area automatically gives a spectrum (Figure 21B) where the two peaks represent the transitions of interest (e.g. PMP-mannose and PMP-$^{13}C_6$-mannose) and the ion counts are a summation of all the scans detected under the chromatogram.

Samples of varying mannose concentrations from 0.5 nM to 1 uM, each containing the same concentration of internal standard (500 nM of $^{13}C_6$-mannose was used throughout this experiment), were analyzed. A standard curve was determined by plotting the ratio of ion counts for mannose to $^{13}C_6$-mannose against the varying mannose concentrations. Both the PMP-mannose and DHZ-mannose standard curves displayed a linear relationship as shown in Figure 22 (p.75).

Establishing a standard curve using an internal standard allow unknown amounts of product investigated in an enzymatic assay to be quantified. For example, by spiking a mixture of unknown concentration of mannose product with a known amount of $^{13}C_6$-mannose as the internal standard, a new ratio of mannose to $^{13}C_6$-mannose can be obtained. By comparing this new ratio to the predetermined concentrations of mannose in the standard curve, this will allow a new mannose concentration to be assigned to the unknown sample. Technically, other monosaccharide products could be quantified in a similar manner using specific internal standards. Furthermore, MRM can be programmed to analyze multiple analytes of interest at the same time and special softwares (e.g. NeoLynx or QuanLynx) can be used for the automatic generation of standard curves to assist product quantification. Unfortunately, the standard curves developed here were never put to use in this research project because of an unpredicted problem that emerged in the development of the enzymatic assays that will be described in the following section.

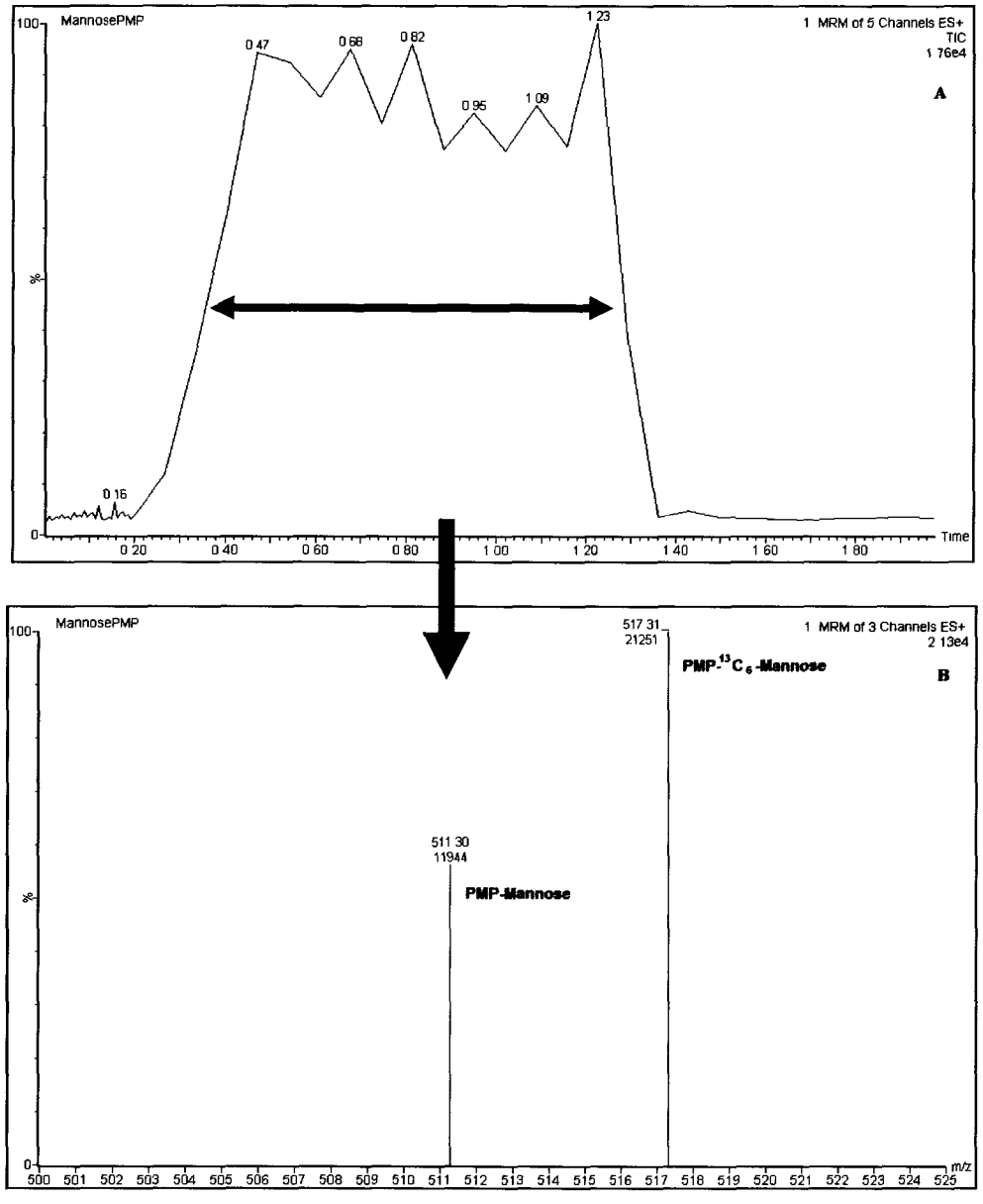


Figure 21. MRM analysis of PMP-mannose and PMP-$^{13}C_6$-mannose.

A) Chromatogram representing the "flow injection analysis" set-up illustrating the time that the sample reaches the detector and the length of time data collected. B) Integrated spectrum representing the summation of the ion counts detected under the chromatogram. Peaks represent the transitions of interest (PMP-mannose and PMP-$^{13}C_6$-mannose). The ratios of mannose to $^{13}C_6$-mannose against the varying mannose concentrations allow a standard curve to be plotted as shown in Figure 22 (p.75).

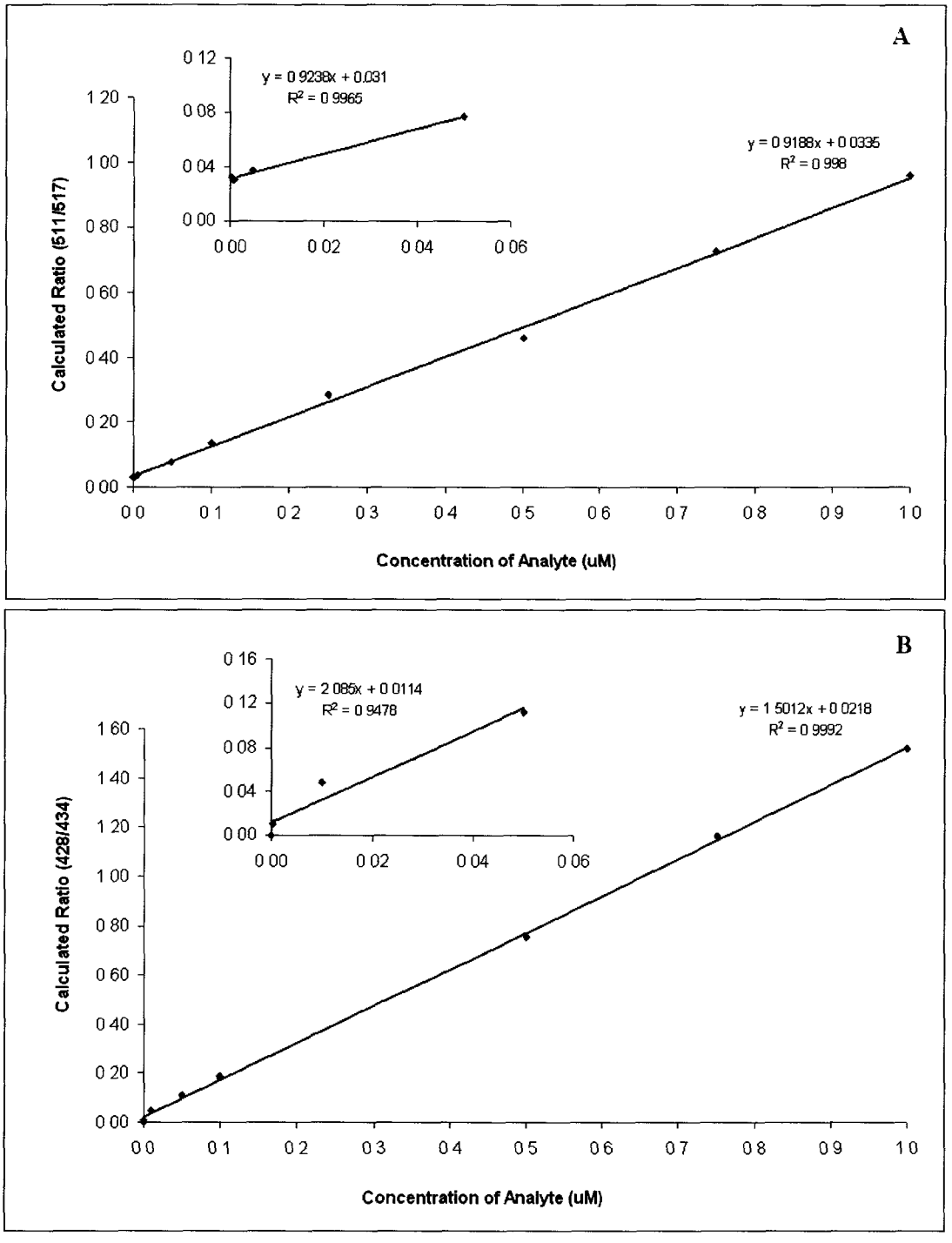


Figure 22. Mannose standard curves for enzymatic product quantification.

The ratios of mannose to $^{13}C_6$-mannose were plotted against varying concentrations of Mannose. A) PMP was used as the derivatizing reagent. B) DHZ was used as the derivatizing reagent.

4.3.4 Attempt to demonstrate exoglycosidase activities by MS/MS

β-Galactosidase and β-Hexosaminidase were the first exoglycosidases studied in this attempt to demonstrate in separate assays that activity could be measured through the appearance of specific monosaccharide products by MS/MS. Assay conditions followed the previous 4MU based fluorometric set-up, but the initial 4MU-monosaccharide substrates were replaced with the new specific disaccharide substrates. The derivatization step could be incorporated into the assay before or after the enzymatic reaction as described earlier, so both approaches were examined.

In the first trial, β-Galactosidase and its target disaccharide, Gal(β1,4)GlcNAc, were tested using the pre-reaction derivatization method where the initial substrate was modified with the addition of PMP before the enzyme source was introduced. Cleavage of the β1-4 linkage resulted in two monosaccharide products, but only GlcNAc contained the original PMP derivative (Figure 23A; p.77). In an attempt to demonstrate β-Galactosidase activity, the appearance of PMP-GlcNAc was monitored in four different reactions which included a control containing no enzyme (50 µl of H_2O was used instead), and three other samples that contained 50 µl, 100 µl and 250 µl of fibroblast lysate (AE-active enzyme). Unfortunately, when the PMP-GlcNAc (m/z=552) was followed among the samples, there was no apparent difference in ion counts as shown in Figure 23B. Even the control sample contained a source of unexpected PMP-GlcNAc. In-source fragmentation at MS1 might explain this unexpected level of PMP-GlcNAc since the initial PMP-Gal(β1,4)GlcNAc substrate contains a weak glycosidic linkage that could easily be broken. A pre-reaction derivatization approach did not seem to allow proper determination of enzyme activity by monitoring a unique monosaccharide product.

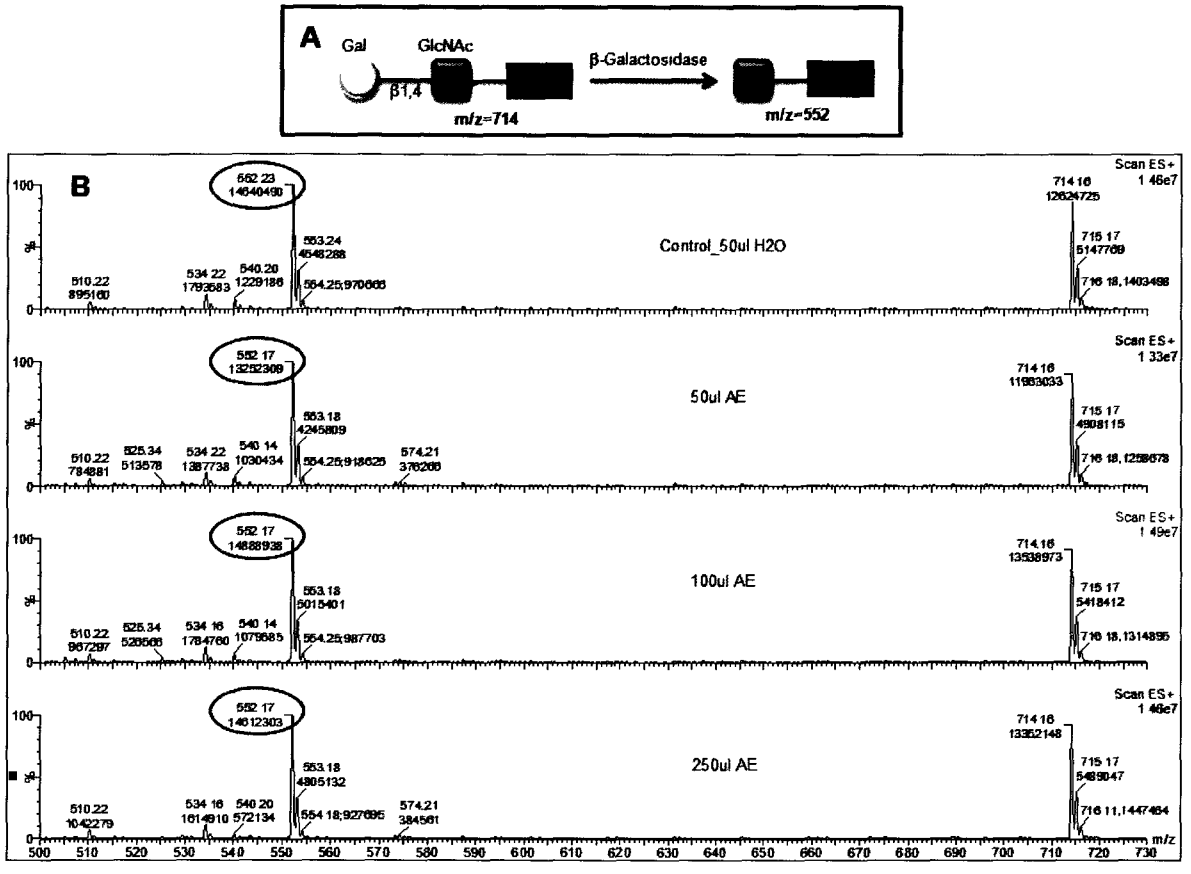
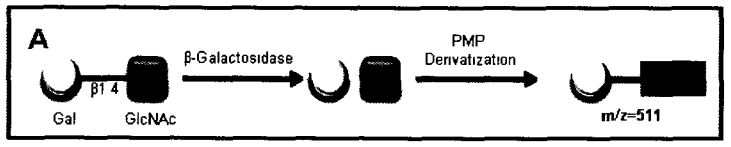


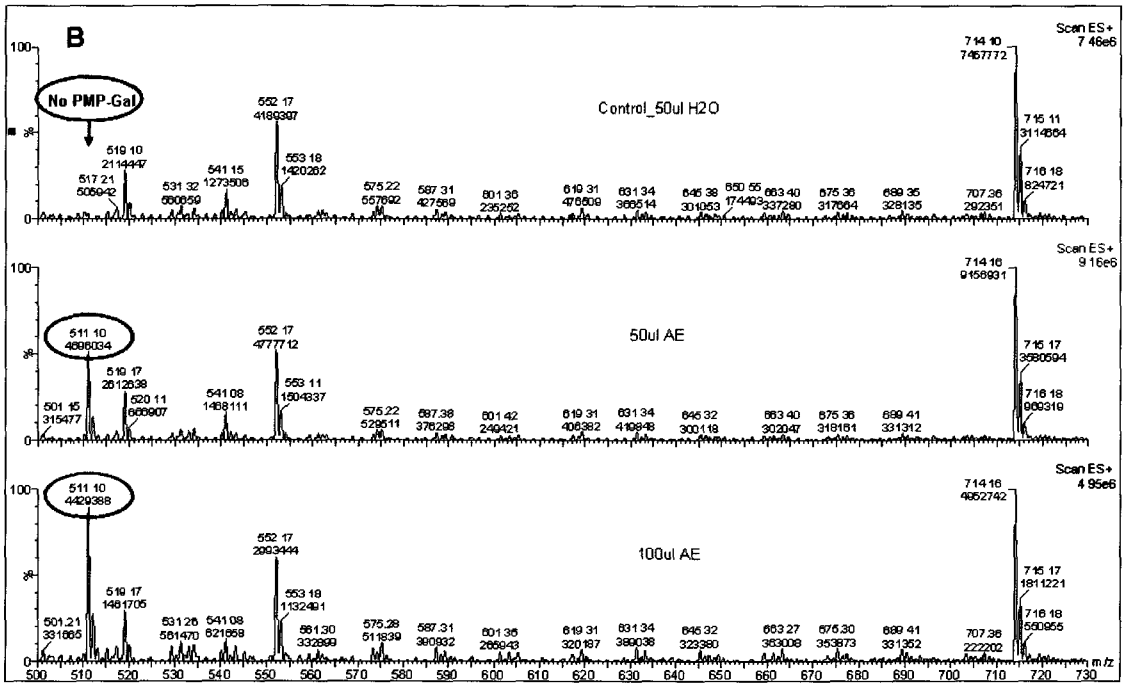
Figure 23. Pre-reaction derivatization method.

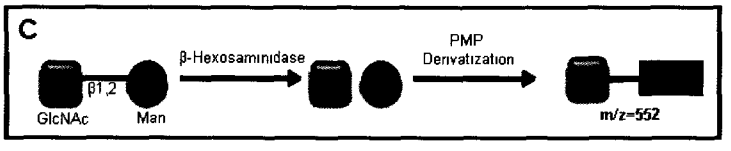
(A) Schematic representation of the pre-reaction derivatization method using PMP and the monosaccharide of interest is PMP-GlcNAc. (B) MS detection of PMP-GlcNAc (m/z=552) after enzymatic reactions. AE (active enzyme) was aliquoted from an enzyme source collected from one 75-cm^2 flask of fibroblasts in culture. This method could not demonstrate β-Galactosidase activity because all samples displayed a similar PMP-GlcNAc signal.

Turning to the post-reaction derivatization method, the enzymatic reaction was carried out first and then PMP was incorporated afterwards. Both Gal and GlcNAc released by β-Galactosidase were expected to contain a PMP. Technically, both monosaccharides could be detected by MS/MS but only monitoring one product would be sufficient to distinguish β-Galactosidase activity (Figure 24A; p.79-80). Reaction samples using the same lysate volumes were analyzed as in the pre-reaction derivatization method. It is important to note that this post-reaction derivatization experiment is independent from the previous pre-reaction derivatization experiment. Fresh fibroblast cells were cultured from separate 75-cm^2 flasks. Thus, the concentration of enzyme within the experiment was the same and differed between experiments. By monitoring PMP-Gal (m/z=511), β-Galactosidase activity appeared to be demonstrated through increasing amounts of PMP-Gal in each sample that contained increasing amounts of lysate (AE-active enzyme) as shown in Figure 24B. The post-reaction derivatization method was also tested for β-Hexosaminidase. The disaccharide GlcNAc(β1,2)Man was used as the starting substrate and PMP-GlcNAc (m/z=552) was monitored to determine β-Hexosaminidase activity (Figure 24C). Again, increasing amounts of PMP-GlcNAc were observed when the lysate volume in each sample was increased as shown in Figure 24D.

Being able to detect two different monosaccharide products separately in two exoglycosidase assays each starting with a unique disaccharide substrate meant that these reactions could be multiplexed for the simultaneous determination of both β-Galactosidase and β-Hexosaminidase activities. Unfortunately, further investigations into this post-reaction derivatization method revealed that these monosaccharide products are not a true reflection of enzyme activities because of contaminating endogenous hexoses in the lysate.


A
β-Galactosidase
PMP
Derivatization
β1 4
Gal
GlcNAc
m/z=511


B
No PMP-Gal
Control_50ul H2O
50ul AE
100ul AE
m/z


C
β-Hexosaminidase
PMP
Derivatization
β1,2
GlcNAc
Man
m/z=552

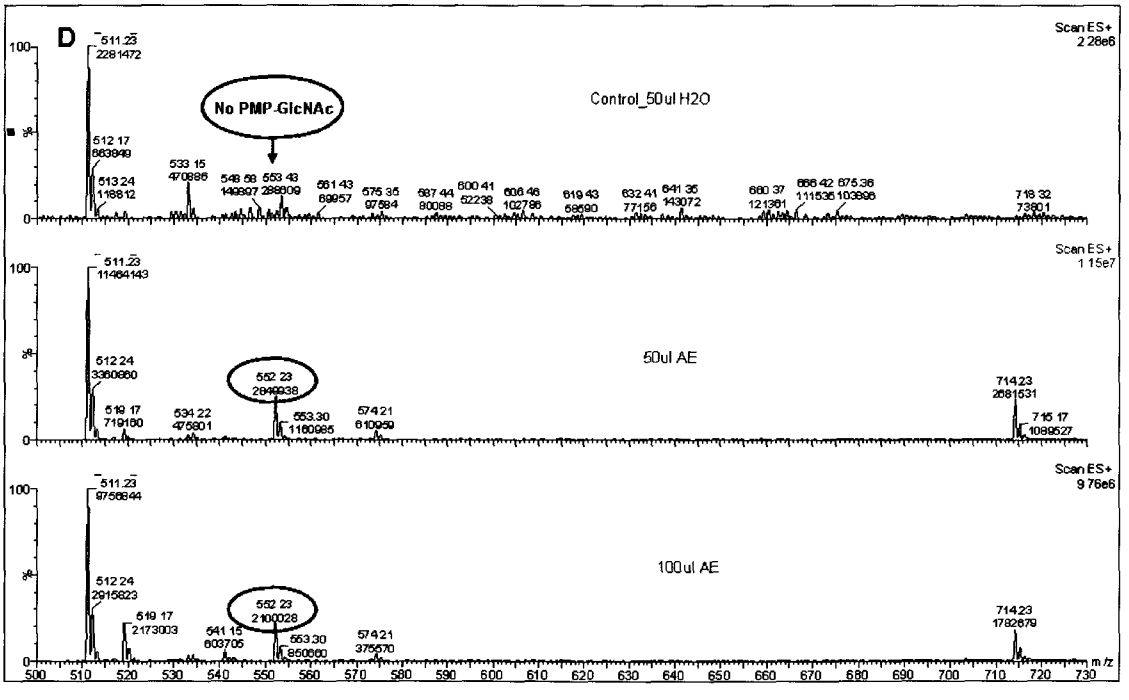
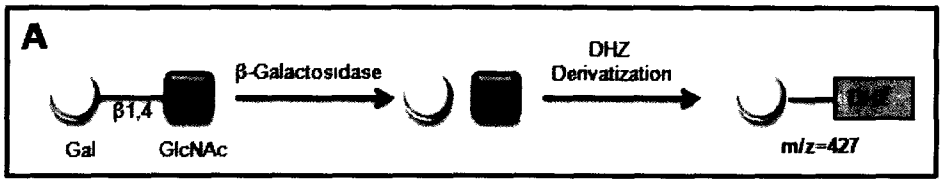

D
No PMP-GlcNAc
Control_50ul H2O
50ul AE
100ul AE
m/z

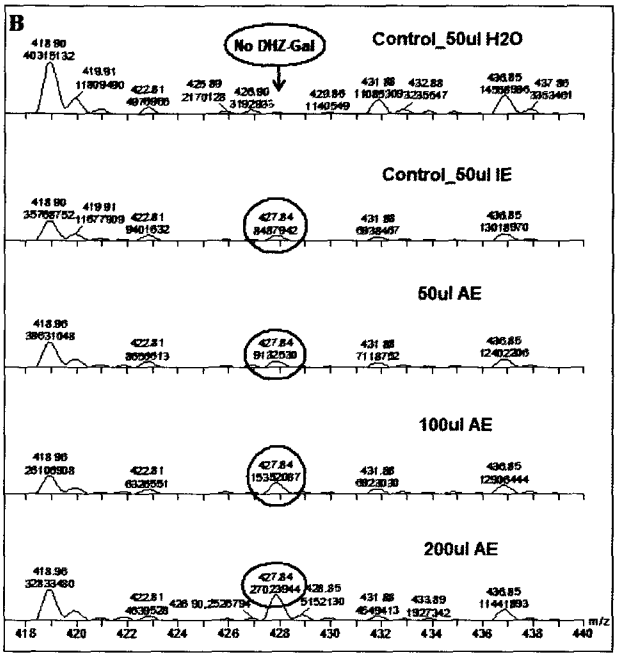
Figure 24. Post-reaction derivatization method.

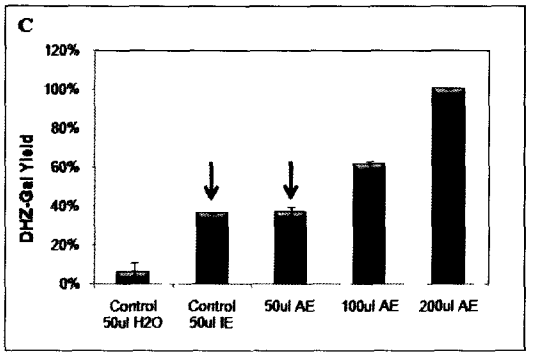
(A) (C) Schematic representation of the post-reaction derivatization method using PMP and the monosaccharides of interest in each reaction are PMP-Gal (m/z=511) or PMP-GlcNAc (m/z=552). (B) (D) MS detection of PMP-Gal or PMP-GlcNAc after enzymatic reactions. AE (active enzyme) was prepared from skin fibroblast cells. β-Galactosidase and β-Hexosaminidase assays are independent.

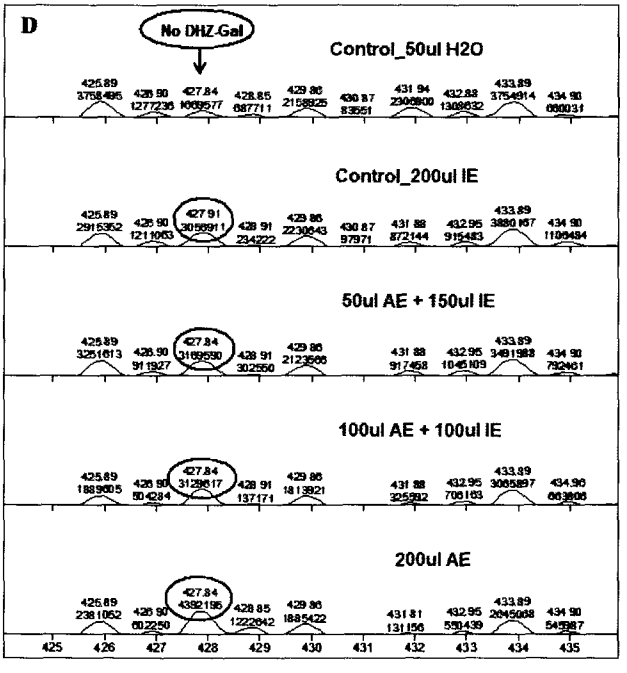
4.3.5 Interfering endogenous hexoses mask exoglycosidase activity

The post-reaction derivatization method was also tested using the DHZ reagent in an attempt to demonstrate β-Galactosidase activity. DHZ-Gal (m/z=427) was the monosaccharide studied (Figure 25A; p.82-83). Careful analysis of this post-reaction derivatization experiment revealed that the DHZ-Gal detected within each sample was not all produced from the initial Gal(β1,4)GlcNAc substrate. In one experiment, a control containing 50 µl of H_2O instead of enzyme was compared to three other samples containing either 50 µl, 100 µl or 200 µl of active enzyme (AE) and by monitoring DHZ-Gal, an increase in the amount of product, hence β-Galactosidase activity, was thought to be observed (Figure 25B,C). However, when a second control was added to this experiment, which contained 50 µl of inactive enzyme (IE) prepared from fibroblast lysate heated at 100°C for 5 minutes, a similar amount of DHZ-Gal was interestingly detected as compared to the sample containing 50 µl AE (Figure 25C, black arrows). In a second experiment, the same five reactions were compared, however in addition to the volume of AE that was present, IE was added to maintain the same lysate volume and protein content. Other than the H_2O control, all samples demonstrated a similar amount of DHZ-Gal (Figure 25D, E, black arrows). Since derivatization was carried out at the end after the enzymatic reaction, it is likely that endogenous hexoses from the lysate, containing a similar molecular weight, might also have incorporated excess DHZ. Therefore, the products detected in Figure 25B,C and previously in Figure 24 (p.79-80) might instead be the total amount of monosaccharide from the lysate, which is masking the real enzymatic product. Interfering isomeric hexoses from the lysate was an unexpected challenge that had to be overcome before further developments to the multiplex assay could be investigated.


A
β-Galactosidase
DHZ
Derivatization
β1,4
Gal
GlcNAc
m/z=427


B
No DHZ-Gal
Control_50ul H2O
Control_50ul IE
50ul AE
100ul AE
200ul AE
m/z


C
DHZ-Gal Yield
120%
100%
80%
60%
40%
20%
0%
Control 50ul H2O
Control 50ul IE
50ul AE
100ul AE
200ul AE


D
No DHZ-Gal
Control_50ul H2O
Control_200ul IE
50ul AE + 150ul IE
100ul AE + 100ul IE
200ul AE

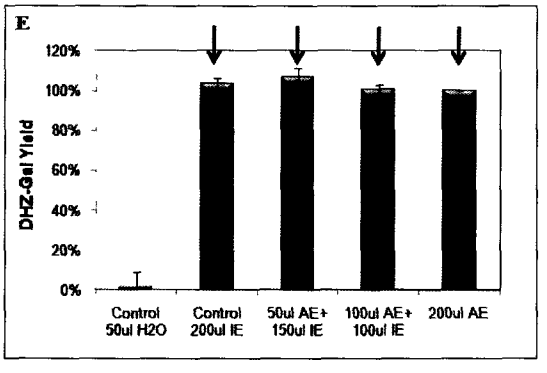

E
DHZ-Gal Yield
120%
100%
80%
60%
40%
20%
0%
Control 50ul H2O
Control 200ul IE
50ul AE+ 150ul IE
100ul AE+ 100ul IE
200ul AE

Figure 25. Endogenous hexoses from lysate masking β-Galactosidase activity.

(A) Schematic representation of the post-derivatization method using DHZ and the monosaccharide of interest was DHZ-Gal. (B) (D) MS detection of DHZ-Gal (m/z=427) after enzymatic reactions. AE (Active enzyme) and IE (Inactive enzyme) were prepared from skin fibroblast cells, IE was heated at 100°C for 5 minutes. (C) (E) DHZ-Gal was monitored and expressed as a percentage of the maximum yield observed in the 200 µl AE sample. Black arrows indicate same level of product detected, which is likely interference from hexoses found in the fibroblast lysate. Results are expressed as the mean ± SE of 2 independent experiments.

4.3.6 Attempt to separate monosaccharide isomers by UPLC-MS/MS

From here, monosaccharides could not be further used for product detection in the exoglycosidase assays unless the monosaccharides within the lysate could be separated or altered to another form to eliminate interference with the product of interest coming from the *in vitro* assay. First, it was beneficial to find out what kinds of hexoses were present in fibroblast lysates. To separate these carbohydrates, especially monosaccharide isomers, a liquid chromatography technique was used. As mentioned before, liquid chromatography is readily compatible with MS/MS for a more sensitive and highly specific method for identifying compounds in a mixture. Liquid chromatography uses a column and solvent delivery system to separate compounds based on chemical characteristics such as hydrophobicity or acidic and basic properties and MS/MS allows only the detection of the compounds of interest usually using the MRM approach. Before investigating the monosaccharides that were present in a fibroblast sample, the UPLC-MS/MS system was tested to see if it was possible to separate pure hexose isomers. Glucose (Glu), galactose (Gal) and mannose (Man) were initially derivatized with DHZ and individually analyzed by UPLC-MS/MS using a C_{18} column. The three monosaccharides displayed unique retention times, however, when the sugars were analyzed in a mixture, Man was separated but Glu and Gal could not be clearly resolved (Figure 26; p.85). Due to the lack of availability of specialized columns for separating carbohydrates, this work was not continued. At the moment, the different types of monosaccharides present in fibroblast lysates have not been identified.

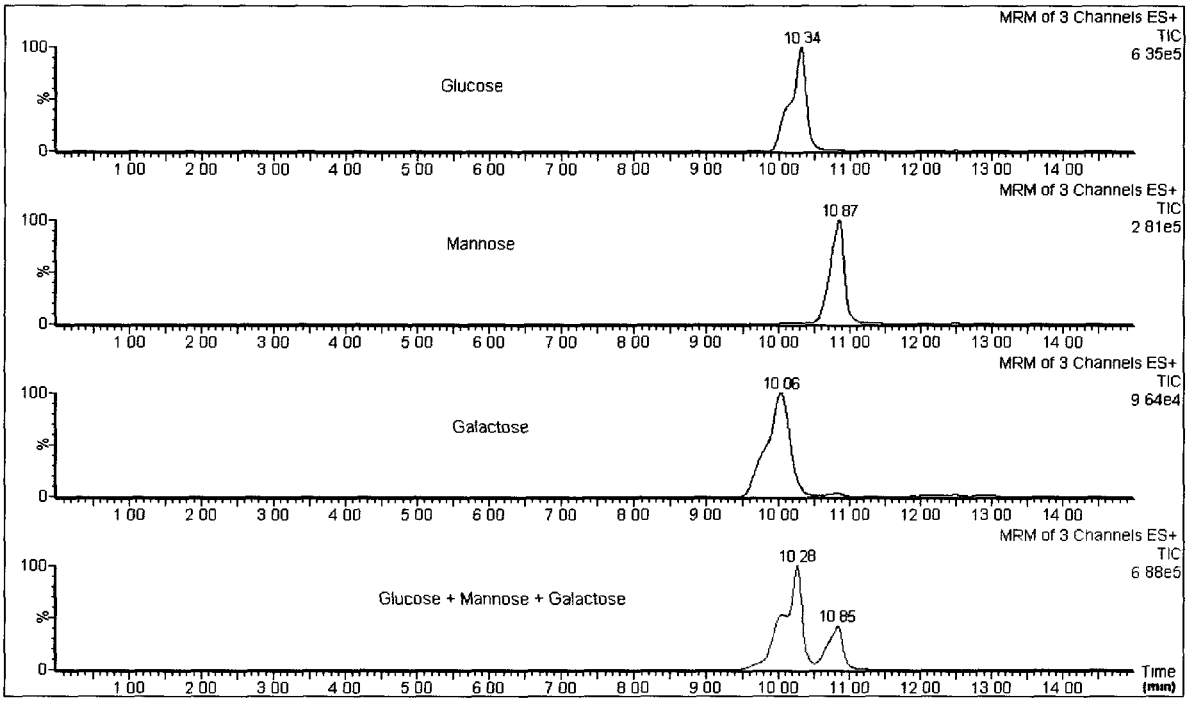


Figure 26. UPLC-MS/MS analysis of monosaccharide isomers.

Pure Glucose, Mannose and Galactose were derivatized with DHZ. Individual chromatograms are displayed in the first three panels. A mixture of the three sugars were examined in the forth panel. Retention times are described at the top of each peak.

4.4 Discussion

In this section, many interesting and unpredicted results were unveiled in an attempt to bridge together the previous exoglycosidase assays using the newly established universal buffer conditions (30 mM citrate-phosphate, pH 4.3) and using MS/MS as a new product detection method. Since carbohydrates are generally neutral compounds, derivatization is often necessary to enhance their ionization. In this part of the study, it was found that PMP and DHZ were good candidates for carbohydrate derivatization to assist MS/MS analysis. When PMP and DHZ derivatization methods were optimized, MS/MS detection of DHZ-sugars significantly exceeded the signal detected for PMP-sugars. Having two reliable carbohydrate derivatives meant that a wider array of natural substrate analogs could be used for designing a multiplex assay that could target the group of exoglycosidases. Multiple enzyme activities could be followed if unique monosaccharide products were present for each enzyme studied. A theoretical multiplex reaction scheme for the exoglycosidases is displayed in Figure 14 (p.59). One major challenge is that many monosaccharide products are isomeric. A potential solution to this problem is to use two derivatization steps that could be incorporated in a pre-/post- enzymatic reaction manner where particular monosaccharide products are selectively tagged with either PMP or DHZ. It is predicted that MS/MS could detect all of the different products as long as there is a mass difference.

Mannose and a correlating internal standard, $^{13}C_6$-mannose, had been investigated to generate a standard curve to be used as a product quantification method. The standard curve was prepared by plotting the mannose to $^{13}C_6$-mannose ratios against varying mannose concentrations. An MRM analysis approach was used for data collection. Of note,

this was the first time two analytes (mannose and $^{13}C_6$-mannose) could be detected within one sample by MS/MS, demonstrating the multiplexing capabilities of the MS/MS technology. Linear relationships were observed for the two mannose standard curves generated with either PMP or DHZ reagents. In theory, by determining a mannose to $^{13}C_6$-mannose ratio from a sample containing unknown amounts of mannose, comparison with the linear standard curve could lead to accurate determination of the mannose concentration. Unfortunately, there was no opportunity to test this quantification method during this research.

The next step was to look into the possibility of developing a new individual exoglycosidase assay using the universal buffer (30 mM citrate-phosphate, pH 4.3), a new disaccharide substrate and incorporating a derivatization step. Enzyme activity was determined if a particular monosaccharide product could be detected by MS/MS. First, pre-reaction derivatization and post-reaction derivatization methods were examined separately using PMP. Since β-Galactosidase activity was not detected in the pre-reaction derivatization method due to in-source fragmentation, no further investigations were done to resolve this issue. In the post-reaction derivatization method, β-Galactosidase activity was initially thought to be observed when increasing amounts of the PMP-Gal product was measured in test samples containing increasing volumes of fibroblast lysate. The post-reaction derivatization method displayed a similar increasing trend when PMP-GlcNAc was followed to demonstrate β-Hexosaminidase activity. Even when DHZ was used as the derivatization reagent in attempt to show β-Galactosidase activity again, an increase in the DHZ-Gal product was also noticed. However, further investigations of this post-reaction derivatization method using DHZ revealed that the monosaccharide products observed

were not reflective of true enzyme activity. A control sample containing IE revealed a similar monosaccharide signal when compared to a sample containing the same volume of AE. Endogenous hexoses from the lysate might be the source of the interfering signal. True monosaccharide products coming from the disaccharide substrate as a result of enzyme activity seemed to be masked by these hexoses from the lysate.

UPLC-MS/MS was tested to try to differentiate pure monosaccharide isomers, but the method was unsuccessful due to inadequate column chemistry. More time to investigate this monosaccharide separation technique might allow the sugars that were found in the fibroblast lysates to be characterized. Furthermore, if endogenous hexoses could be separated from the monosaccharide product coming from the target exoglycosidase reaction by UPLC-MS/MS, this technique might be an even greater detection method for future development of an exoglycosidase multiplex assay in analyzing multiple monosaccharide products due to the increased sensitivity and specificity of the system. At this point, another approach was taken to try to demonstrate exoglycosidase multiplexing using MS/MS, excluding UPLC.

PART D: MS/MS multiplexing with 4MU and PNP substrates

5.1 Introduction

Paranitrophenol (PNP)-monosaccharide substrates resembling the 4MU-monosaccharide substrates are commercially available and the linkages between the monosaccharides and the PNP moiety are also recognized by unique exoglycosidases. The amount of PNP produced by a particular enzyme in the presence of a corresponding substrate can be measured in the same manner as detecting 4MU products in the previous 4MU based exoglycosidase assays, but a spectrophotometer is generally used for detection instead. PNP products are identified at about 400 nm, allowing enzyme activity to be measured in a given biological sample. Theoretically, if a PNP-monosaccharide and a 4MU-monosaccharide substrate were present in the same reaction tube, two different exoglycosidase activities could be assayed in a multiplex manner by detection of PNP and 4MU products (Figure 27; p.90). MS/MS could still be used as the detection system since PNP and 4MU contain unique functional groups that are readily ionizable by ESI and they contain different masses that will allow them to be detected as two separate entities. Since PNP and 4MU are not found in biological samples, this will resolve the issue of measuring interfering endogenous hexoses from the fibroblast lysates. Detecting PNP and 4MU will demonstrate real exoglycosidase activity coming only from the artificial substrates. The only downfall to working with these types of compounds is that the linkages between the monosaccharide and the PNP or 4MU are not true natural glycosidic linkages as they are found in natural disaccharide substrates.

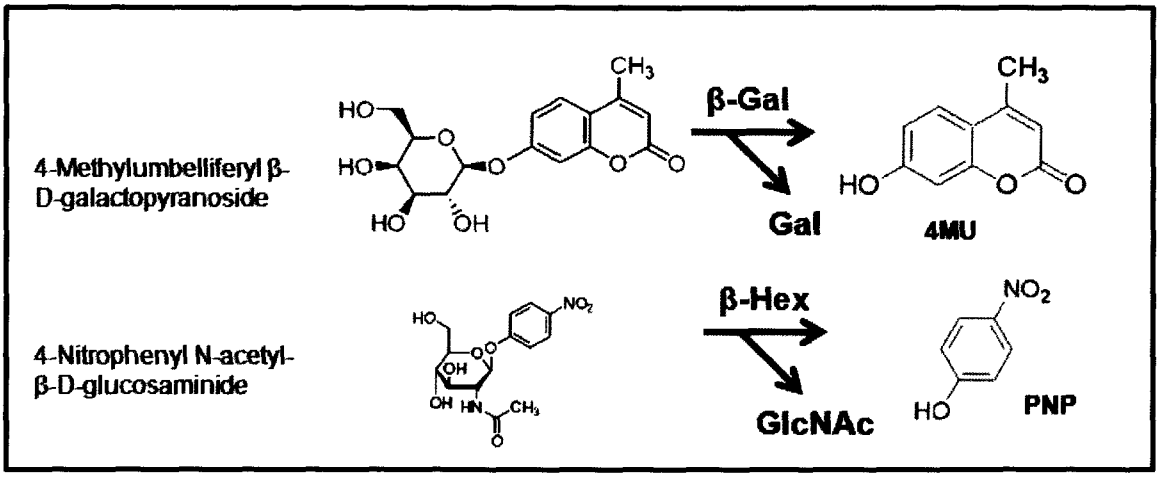


Figure 27. Multiplex assay using 4MU and PNP substrates.

4-Methylumbelliferyl-β-D-galactopyranoside is a target substrate for β-Galactosidase and 4-nitrophenyl N-acetyl-β-D-glucosaminide is a target substrate for β-Hexosaminidase. A multiplex assay could be generated for determining β-Galactosidase and β-Hexosaminidase activities by MS/MS through the detection of released 4MU and PNP products from the same reaction tube.

5.2 Materials and Methods

β-Galactosidase and β-Hexosaminidase assays were set-up in a similar manner to previous methods (Table 3; p.29). 4-Methylumbelliferyl-β-D-galactopyranoside (Sigma Aldrich, Canada) and 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma Aldrich, Canada) were respectively used as the initial substrates to target the determination β-Galactosidase and β-Hexosaminidase activities through the detection of either 4MU or PNP products. The universal buffer (30 mM citrate-phosphate, pH 4.3) was used and the reactions were incubated for 2 hours and then boiled for 5 minutes to stop the enzymatic reactions. Content was dried *in vacuo* for 2.5 hours and 500 µl of CH_3CN was used for product extraction. Samples were centrifuged at 13,200 rpm for 2 minutes and the supernatant was dried. Residue was reconstituted in [(80:20 CH_3CN: H_2O) + 0.1 % CH_2O_2 + 2 mM NH_4OAc] and 10 µl was injected for analysis by MS/MS. When β-Galactosidase and β-Hexosaminidase were analyzed separately, reactions were run in separate test tubes and when they were multiplexed, the reactions were set up in the same test tube.

5.3 Results

5.3.1 Detection of 4MU and PNP compounds by MS/MS

Since 4MU and PNP contain ionizable functional groups, prior derivatization steps were not necessary. However, the differing ionizable groups found between these molecules meant that separate ionization modes had to be investigated for determining the optimal signal for each of these compounds. Generally, electrospray ionization (ESI) can be accomplished in the positive mode (ESI+) which produces positive ions or in the negative mode (ESI-) which produces negative ions. MS/MS analysis revealed that 4MU was detectable in both the ESI+ (m/z=177) and ESI- (m/z=175) modes, however ESI+ produced a higher 4MU signal (Figure 28A; p.93). PNP was only visible in the ESI- (m/z=138) mode (Figure 28B). Two new P/D transitions were further identified for 4MU (177>77) and PNP (138>108). A new flow injection analysis using an MRM scanning technique was programmed using both ESI+/- modes for the simultaneous detection of these two compounds to allow multiplexing by MS/MS.

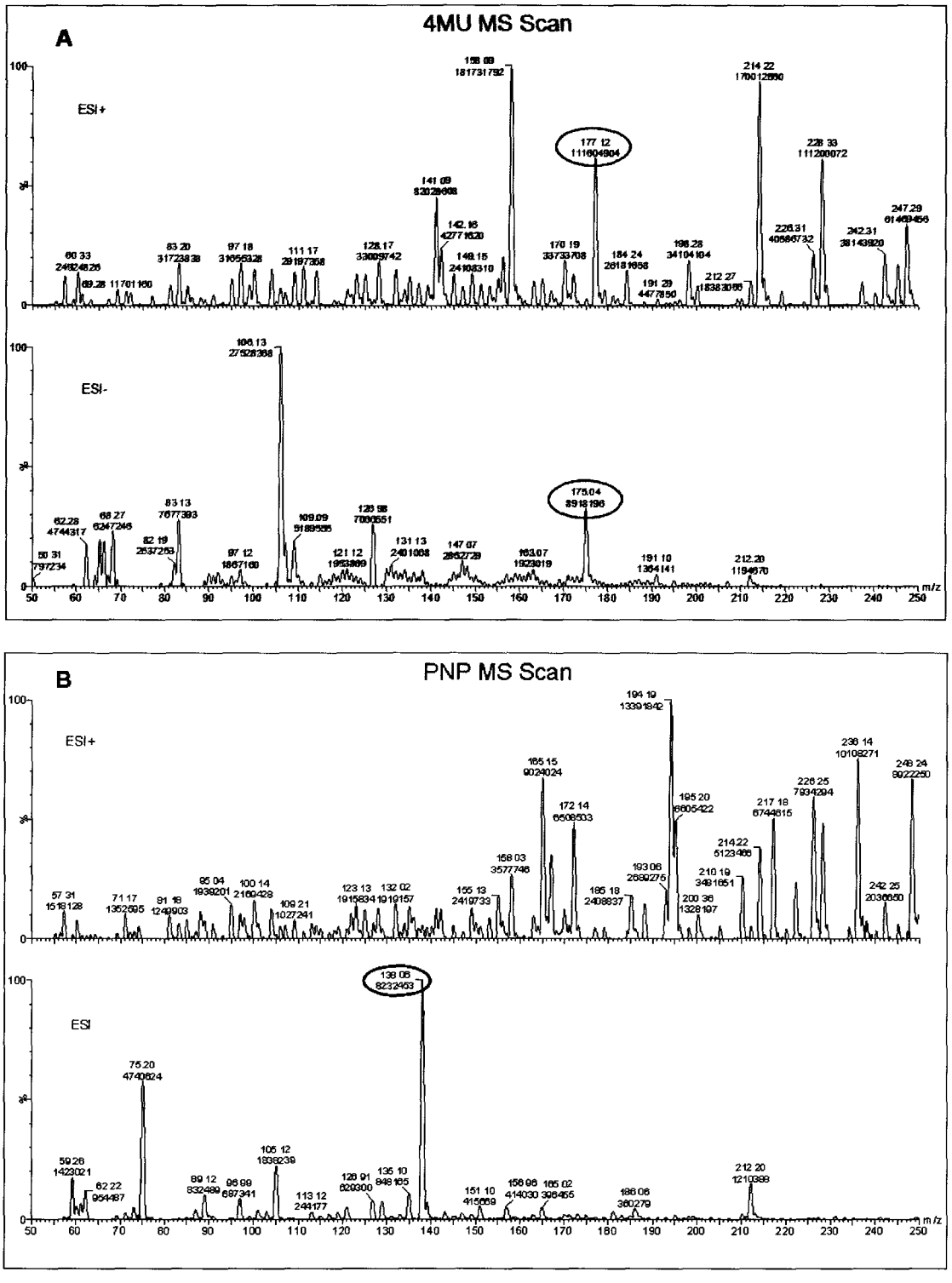


Figure 28. Detection of 4MU and PNP by MS/MS.

Pure 4MU and PNP were analyzed by MS/MS. (A) 4MU was detectable under ESI+ (m/z=177) and ESI- (m/z=175). (B) PNP was only detectable under ESI- (m/z=138).

5.3.2 β-Galactosidase and β-Hexosaminidase activities in separate assays

Initially, β-Galactosidase and β-Hexosaminidase reactions were run in parallel in separate test tubes and the corresponding 4MU or PNP products were monitored by MS/MS using the appropriate ESI modes. To demonstrate enzyme activity, both the β-Galactosidase and β-Hexosaminidase assays compared 5 different reaction samples that contained increasing amounts of AE from fibroblasts (25, 50, 100, 150, and 200 µl) to a control with 200 µl IE, which was fibroblast lysate that had been heat inactivated. All samples were brought to the same final volume of 200 µl using IE. Activities in each of the β-Galactosidase and β-Hexosaminidase assays were monitored separately by MS/MS detection of either 4MU or PNP products. Interestingly, both assays showed an increasing trend when 4MU and PNP were plotted against increasing amounts of AE (Figure 29). 4MU and PNP were expressed as a percentage of the highest signal found in the 200 µl AE sample. Interestingly, *in vitro* β-Galactosidase and β-Hexosaminidase activities could be determined without interference from the fibroblast lysates.

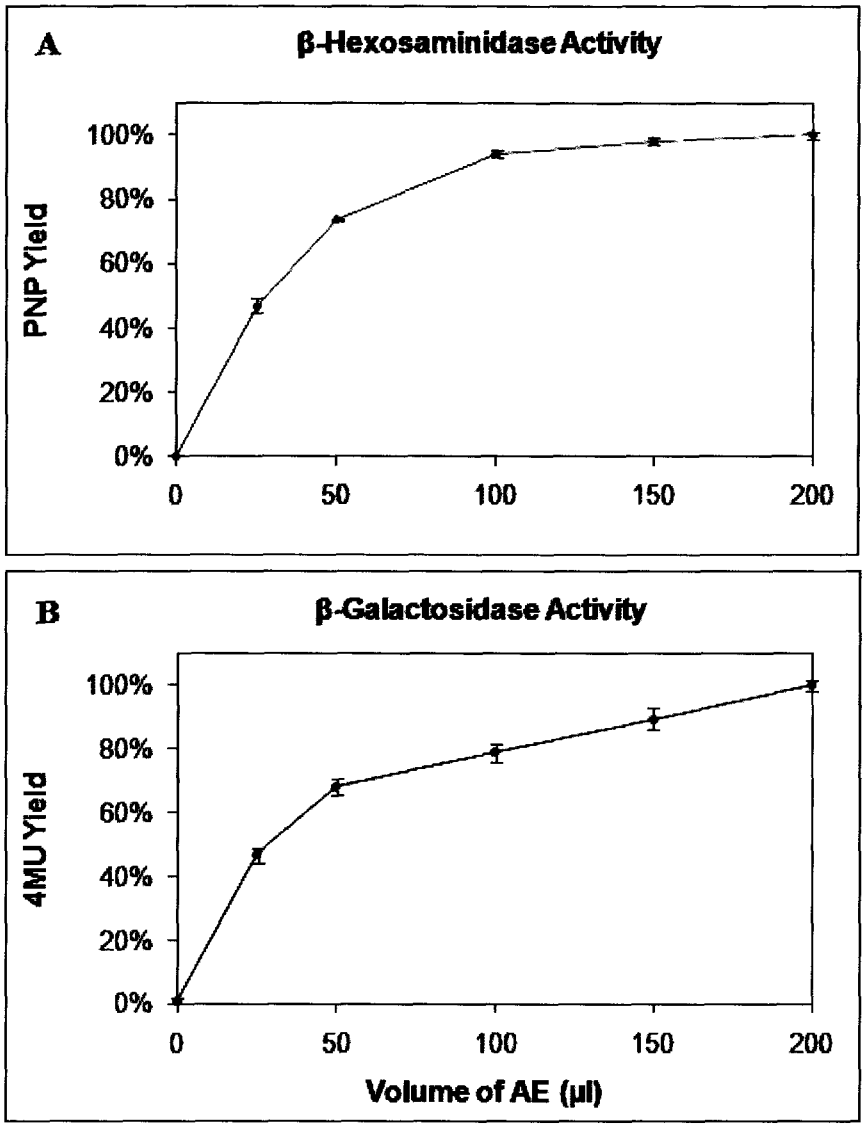


Figure 29. MS/MS detection of β-Galactosidase and β-Hexosaminidase activities in separate assays.

The enzyme source was from whole cell fibroblast lysates. AE (active enzyme) represents untreated lysate. IE (inactive enzyme) had been heated for 5 minutes at 100°C. 4MU and PNP were monitored individually by MS/MS and each product was expressed as a percentage of the maximum signal observed in the 200 µl AE sample. β-Hexosaminidase activity is demonstrated in A) and β-Galactosidase activity is demonstrated in B). Results represent the mean ± SE of 2 independent experiments.

5.3.3 β-Galactosidase and β-Hexosaminidase activities in a combined assay

A multiplex assay was prepared for β-Galactosidase and β-Hexosaminidase analysis in the same reaction tube, and activities were monitored by MS/MS through the detection of both 4MU and PNP products from a single sample injection. 5 samples containing increasing amounts of AE (25, 50, 100, 150, and 200 µl) were compared to a control containing 200 µl IE. All samples were brought to the same final volume of 200 µl using IE. Both the 4-methylumbelliferyl-β-D-galactopyranoside and 4-nitrophenyl N-acetyl-β-D-glucosaminide substrates were present in all the samples to target both β-Galactosidase and β-Hexosaminidase activities at the same time. Interestingly, an increasing trend was observed when both 4MU and PNP products were plotted against increasing amounts of AE (Figure 30; p.98). This was the first successful trial that demonstrated the multiplexing potential of MS/MS to detect multiple exoglycosidase activities in a single assay.

These β-Galactosidase and β-Hexosaminidase assays performed individually or in combination demonstrated a hyperbolic effect in the amount of product produced as more lysate was added in each sample. Several explanations could explain this non-linear relationship including substrate depletion, product inhibition or oversaturation of the detection capacity of the MS/MS detector. Clearly, enzyme kinetic studies investigating kinetic properties such as the rate at which the enzyme becomes saturated with a particular substrate and maximum reaction rates need to be examined to obtain an optimal set of conditions to allow further development of a more precise multiplex assay. Initially, to determine the limits of detection for the MS/MS system, a standard curve of pure 4MU and PNP was analyzed. Lowest limit of detection for 4MU or PNP was found in the nM range and linearity was maintained up to 100 µM for both analytes (Figure 31; p.99). 4MU or

PNP products detected from the β-Galactosidase and β-Hexosaminidase assays using AE samples that contained up to 200 µl of active lysate were still within this linear range. A time course experiment of up to 6 hours was further investigated to re-determine the optimal incubation times for β-Galactosidase and β-Hexosaminidase assays. Linearity of both 4MU and PNP products was maintained only within the first hour of incubation (Figure 32; p.100). This experiment demonstrated that a shorter incubation time (half hour) would be sufficient to obtain optimal activities for both β-Galactosidase and β-Hexosaminidase using the 4-methylumbelliferyl-β-D-galactopyranoside and 4-nitrophenyl N-acetyl-β-D-glucosaminide substrates. In further establishing a multiplex assay for the remaining exoglycosidases, new substrates need to be identified and several assay parameters will have to be re-optimized.

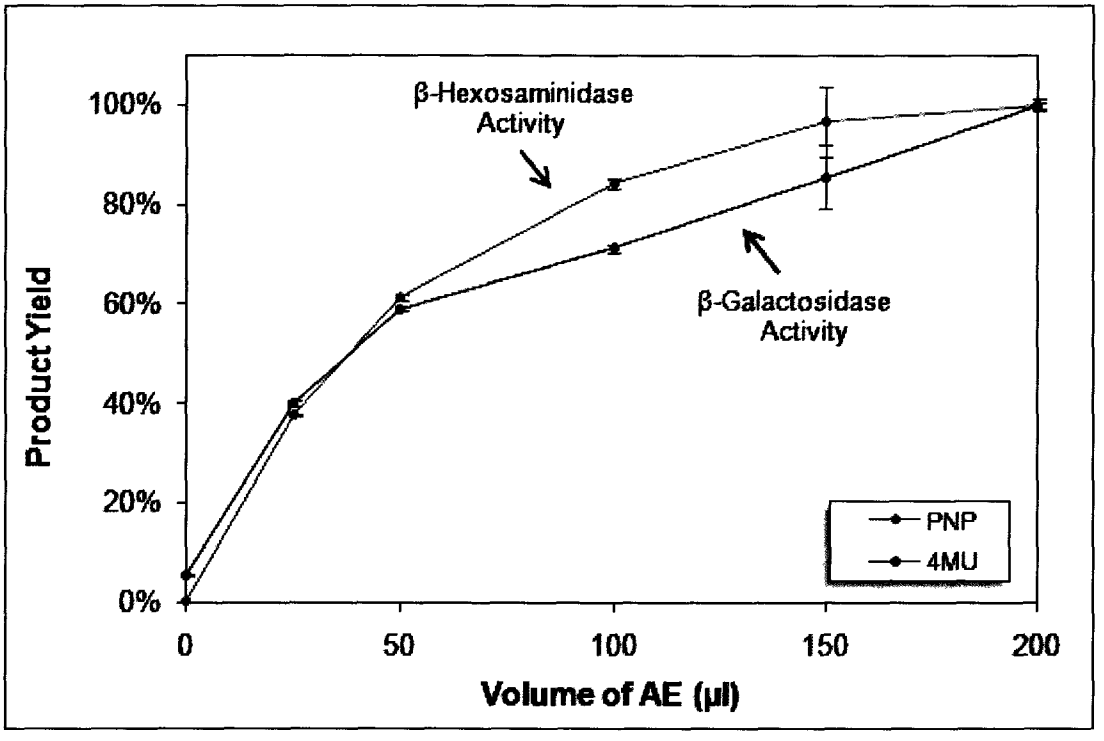


Figure 30. MS/MS detection of β-Galactosidase and β-Hexosaminidase activities in a multiplex assay.

The enzyme source was from whole cell fibroblast lysates. AE (active enzyme) represents untreated lysate. IE (inactive enzyme) had been heated for 5 minutes at 100°C. 4MU and PNP were monitored together by MS/MS and each product was expressed as a percentage of the maximum observed in the 200 µl AE sample. Results represent the mean ± SE of 2 independent experiments.

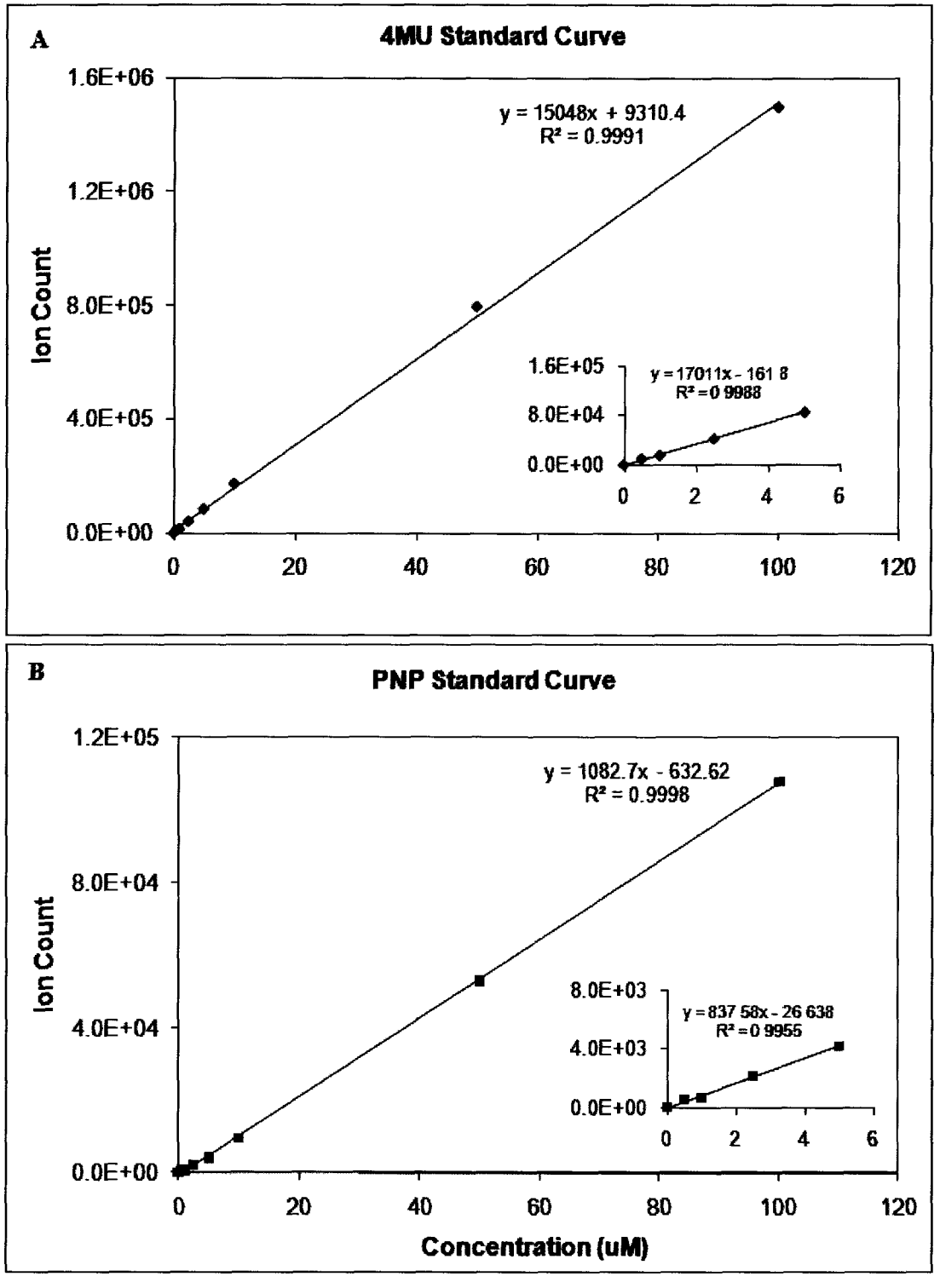


Figure 31. 4MU and PNP standard curves in a MS/MS system.

4MU (A) and PNP (B) ion counts were plotted against varying concentrations of 4MU and PNP. Linearity was observed up to 100 μM of 4MU or PNP.

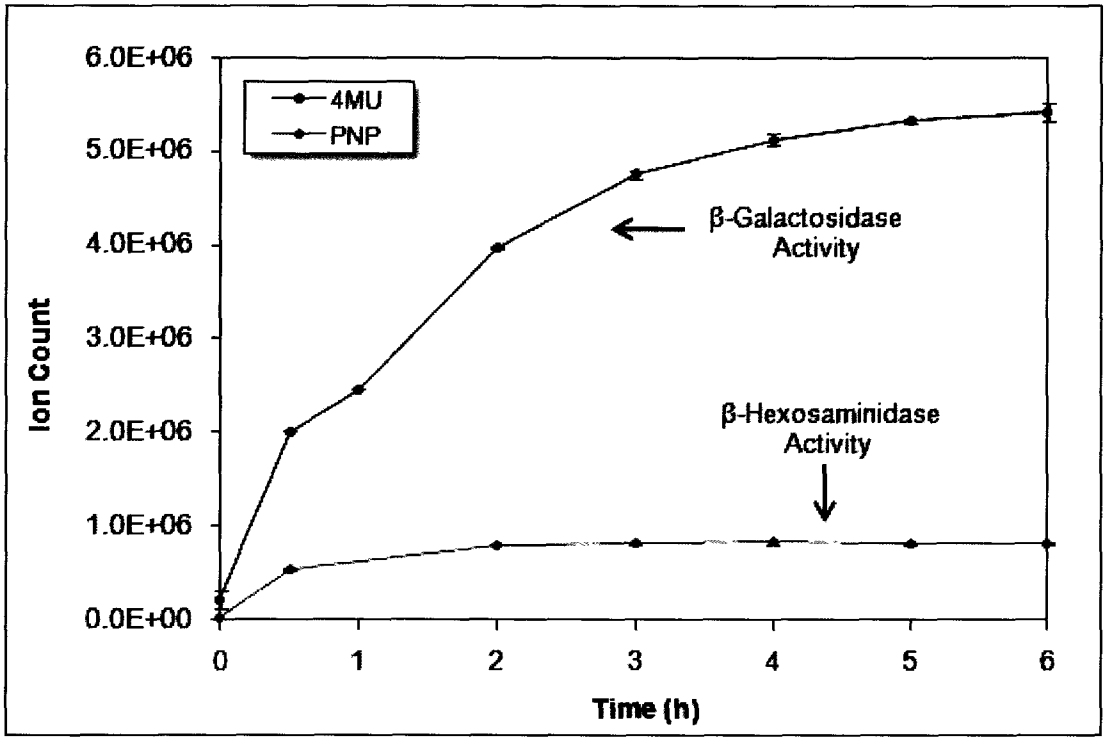


Figure 32. Enzyme activities measured by 4MU and PNP as a function of incubation time.

All samples contained 200 µl AE. The enzyme source was from whole cell fibroblast lysates. Each time point was carried out in duplicates. The error bars represent the mean ± SE.

5.4 Discussion

In accomplishing the last goal of demonstrating the ability to multiplex for at least two exoglycosidases (β-Galactosidase and β-Hexosaminidase) using a MS/MS detection approach, the 4MU-based fluorescence substrates were re-visited and a new type of artificial substrate containing a PNP moiety was tested. These are not the ideal substrates to work with because the linkage between the monosaccharide and PNP or 4MU are not glycosidic links between two monosaccharides as found naturally in disaccharide substrates. However, by measuring 4MU and PNP, which are released by target exoglycosidases, meant that monosaccharides do not have to be evaluated as products. Hence, endogenous hexoses coming from the lysate should not interfere with detection.

Interestingly, a successful multiplex assay was established for determining β-Galactosidase and β-Hexosaminidase activities by detecting both PNP and 4MU products simultaneously using MS/MS. Standard curves of pure PNP and 4MU demonstrated strong linear correlations, up to 100 μM of analytes could be efficiently detected from this system. This proof of concept experiment used 4MU assay conditions that were based on previous fluorescence detection methods, but MS/MS was used for product analysis instead. These experiments only demonstrated that exoglycosidase multiplexing using MS/MS as a detection system is possible. Without a doubt, each assay needs to be carefully investigated to re-establish optimal assay conditions. Queries about substrate depletion or product inhibition leading to the current trends will require more experiments to resolve. Studying the enzyme kinetics for these exoglycosidases to measure the reaction rates and understanding the effects of varying the conditions of the reactions are essential to predicting how much the enzymes will react over a certain amount of time and the required

concentrations of a certain compound to have it react at a specified rate. Most enzyme kinetic studies concentrate on the initial, approximately linear part of the enzyme reaction. Even if such tests had been carried out before, any changes in assay conditions, such as substrate, cofactor, or enzyme concentration, pH or temperature, may also cause a deviation in linearity. In following the progress of the reactions in a time course experiment, small digressions in the linear range were obvious when MS/MS was compared to previous fluorescence detection methods. Thus, it will be necessary to re-define standard conditions that are more specific and will hold for all enzymes of interest under MS/MS analysis methods before proceeding with further developments in this multiplex assay.

Once optimal assay parameters are determined for all of the lysosomal enzymes in study, multiplexing by combining the assays as demonstrated for β-Galactosidase and β-Hexosaminidase should easily follow. MS/MS can be used to monitor multiple end products as long as there is a mass difference and enzyme activities should be straightforward to calculate. Of note, a major advantage of this method is that the PNP and 4MU molecules do not require prior derivatization for MS/MS detection. Identification of similar substrates targeting the remaining exoglycosidases that would yield unique differentiable products by MS/MS would be the next challenge in this research.

General Discussion

6.1. Significance of results

The focus of this research had been to develop a multiplex exoglycosidase assay based on newborn screening technology to facilitate the clinical diagnosis of Oligosaccharidoses with the future goal of implementation into a newborn screening program. The ideal method involves rehydrating DBS to release lysosomal enzymes, which when reacted with a set of unique substrates, will result in accumulation of specific products. MS/MS can allow simultaneous measurements of these products and with the aid of mass differentiated internal standards, exoglygosidase activities could be determined at the same time.

Previously, it was shown that lysosomal hydrolases in DBS were very stable and that an enzyme source could be easily collected for enzyme analysis. The detection of lysosomal enzyme activities in DBS faces the problem of low protein content, hence low concentrations of enzymatic products were expected. Two main questions raised from the start were how much exoglycosidase activities could be detected in DBS using previous 4MU fluorescence based enzyme assay conditions (Table 3; p.29) compared to other sample types and what was the difference in activity levels among the different exoglycosidases. Initially, various biological sample types including leukocytes, fibroblasts and DBS were investigated and interestingly exoglycosidase activities, with the exception of α–Neuraminidase, were detected. DBS showed very low enzyme activity levels, therefore, a time course experiment was carried out which indicated that extra incubation time was required to get sufficient activity to characterize each exoglycosidase function. In all sample types, β-Galactosidase and β-Hexosaminidase were more robust than α/β-

Mannosidase and α-Fucosidase in yielding higher activities. β-Galactosidase and β-Hexosaminidase might be more abundant because they are exoglycosidases needed during earlier steps of the N-glycan catabolic pathway (Thomas 2001). From these results, DBS could still be used as a sample type in the development of a multiplex assay as long as the enzymatic reactions are carried out to a time point which does not reflect substrate depletion and detectable activity is still within the linear range of the detection system. These time points have been established for each exoglycosidase in study under the fluorescence detection methods but in using MS/MS as a new detection system, these incubation times need to be re-established. Furthermore, suitable DBS quality control materials are already being developed for laboratories performing LSD screening and good material linearity has been reported (De Jesus et al. 2009). DBS are readily available from screening laboratories and should be further investigated for its use as the enzyme source in the development of an Oligosaccharidoses multiplex assay.

A universal buffer condition (30 mM citrate-phosphate, pH 4.3) was also identified for these exoglycosidases using the 4MU fluorescence based assay methods. Finding a universal buffer meant that exoglycosidases could be further examined under the same *in vitro* environment which greatly supported the rest of this research in attempt to generate a multiplex assay for the group of lysosomal hydrolases. More intriguingly, this kind of experimental approach to find a common buffer by comparing enzyme activities under various buffer conditions had never been described before. This universal buffer was successfully used throughout this research. Its benefits were particularly demonstrated in the final multiplex assay for β-Galactosidase and β-Hexosaminidase when the two assays were set-up in the same reaction tube containing this universal buffer and end products

were readily measured by MS/MS. Similar results should be achievable when other exoglycosidases are incorporated into this multiplex assay containing the universal buffer. Of note, attempting to generate a multiplex assay this way in which one reaction tube can allow the simultaneous reaction and detection of multiple enzymes is, in fact, a different approach taken than the previous LSD multiplex method developed by Gelb et al. (Li et al. 2004b, Gelb et al. 2006). In their assay, separate incubations needed different buffers and multiplexing was only accomplished after enzymatic reactions were completed. The samples were combined at the end prior to MS/MS analysis. In the current research, all enzyme reactions and product detection could hypothetically be carried out in a single tube. Evidently, this method would be more convenient to carry out.

Seeing that the 4MU assays cannot be combined for multiplexing using a fluorescence detection method, a new strategy was devised using alternative substrates so that the end products differed by mass and could be differentiated by MS/MS, allowing multiple enzyme activities to be determined at the same time. Disaccharides containing the glycosidic linkage found in the natural N-glycans were initially selected as precursors and the resulting monosaccharides with differing masses were targeted as products. Assays were set-up in a similar manner to the previous 4MU assays, but disaccharides were used instead as the starting substrate. The monosaccharide products could not be detected by MS/MS on their own, so a derivatization protocol was incorporated either at the beginning where the derivative was added to the disaccharide or at the end, where the monosaccharide products were derivatized. In the pre-reaction derivatization method, when the derivatized disaccharide was exposed to an enzyme source, only one monosaccharide product would theoretically contain the artificial label. Following the appearance of this

product by MS/MS should allow enzyme activity to be measured. Unfortunately, this method was unsuccessful because of in-source fragmentation where an unexpected level of derivatized monosaccharides was present even in a control sample. Post-reaction derivatization was also tested where the predicted outcome were two derivatized monosaccharide products. By following one product, a particular exoglycosidase activity could be distinguished. In the first few trials testing the post-reaction derivatization method, a difference in the level of products was observed between a control sample without an enzyme source and samples that had increasing amounts of active enzyme. However, when a new sample containing heat inactivated lysate was added to this experiment, a similar level of monosaccharide was detected as compared to a sample with the same volume of active enzyme. This meant that monosaccharide products initially detected might not all be derived from the disaccharide substrate. True exoglycosidase activity may be masked by the endogenous level of monosaccharides likely present in the fibroblast lysate. Chromatography was set-up in tandem with MS/MS in attempt to separate these hexose isomers and to determine the types of contaminating monosaccharides in the lysate. However, to date, this has not been resolved due to the lack of specialized columns for carbohydrate analysis. If UPLC-MS/MS becomes available, this technique will be a more sensitive and powerful method to multiplex enzymatic products. On another note, successful derivatization methods to assist MS/MS detection of carbohydrates have been identified in this research. A product quantification method has also been established that can allow monosaccharide product concentrations to be determined using specific internal standards (e.g. $^{13}C_6$-mannose). If the hexoses found in the lysate could be reduced, the reaction scheme devised in Figure 14 (p.59) using multiple derivatives could still be put

forward and the new product quantification method could be integrated to allow enzyme activity determination in a multiplex manner.

Despite the challenges that have been encountered, a multiplex MS/MS method was demonstrated for β-Galactosidase and β-Hexosaminidase. Exoglycosidase activities were measured from a biological fibroblast sample by detecting 4MU and PNP products in a single assay released from the specifically selected 4-methylumbelliferyl β-D-galactopyranoside and 4-nitrophenyl N-acetyl-β-D-glucosaminide substrates. This proof of principle experiment can be expanded to include the remaining exoglycosidases. A quantitative multiplex assay using DBS as the sample type can still be accomplished as long as new starting substrates yield unique end products that can be differentiable by MS/MS. To date, there is no literature reporting the study of analyzing 4MU and PNP by MS/MS. For the first time, it was demonstrated that these commercially available substrates could be used in the same reaction assay to obtain mass differentiable products where MS/MS was able to distinguish by a single analysis.

6.2. Future directions

Having demonstrated that MS/MS can be used to measure the activities of β-Galactosidase and β-Hexosaminidase through the detection of both 4MU and PNP products in the same reaction tube meant that the concept of multiplexing enzyme assays from the start of the reaction where all target substrates are available to a single enzyme extract, through to the end where all enzymatic products are analyzed at the same time by MS/MS, is a valid method to develop a new diagnostic or screening test for the Oligosaccharidoses. To ensure that assays are operating within a linear range, further studies into the enzyme kinetics for β-Galactosidase and β-Hexosaminidase are necessary to establish more precise

standard conditions for individual assays prior to multiplexing. Initially, the incubation time parameter had been re-examined for both of these exoglycosidases, and typical progress curves were generated demonstrating the course of each enzyme reaction. From a progress curve, taking the slope of the initial rate (linear range) will allow the initial rate of reaction, *v,* to be determined. Generally, most lysosomal enzymes, follow the Michaelis-Menten kinetic model of an irreversible single-substrate reaction (Bisswanger 2004, Cook, Cleland 2007). To verify that β-Galactosidase and β-Hexosaminidase kinetics fit this model, the Michaelis-Menten equation ($v = V_{max}[S] / K_M+[S]$) can be used to describe how the slope determined from a progress curve varies with the concentration of substrate. A relatively simple, yet informative experiment to examine enzyme kinetics and re-determine more accurate enzyme assay parameters is to plot a curve of reaction rate (v) against varying amounts of substrate ([S]) to determine V_{max}, which is the maximum velocity before an enzyme becomes saturated with a particular substrate and also to determine K_M (Michaelis constant), defined as the concentration at which the rate of the enzyme reaction is half V_{max}. The plot should yield a hyperbolic saturation curve. Because of this relationship, [S] for studying Michaelis-Menten kinetics should be chosen within the range of the K_M. Knowing these properties suggests how the enzyme will respond to changes in under the defined conditions. After re-determining the optimal incubation time and substrate concentrations for each enzyme assay, a study for finding the appropriate amount of enzyme concentration will be useful to ensure the assay is always in substrate excess. Commercially available pure β-Galactosidase and β-Hexosaminidase might assist in generating more accurate data. Product appearance should be about 100 to 1000 folds less than the amount of the starting substrate, therefore constructing standard curves of pure

analytes resembling the expected products, as established for 4MU and PNP, will allow determination of limits of detection for the MS/MS system. For each of the remaining exoglycosidases of interest, a new substrate like 4-methylumbelliferyl β-D-galactopyranoside for β-Galactosidase and 4-nitrophenyl N-acetyl-β-D-glucosaminide for β-Hexosaminidase, containing a unique conjugate to a monosaccharide, could potentially be used to develop other enzyme assays as long as the generated product is differentiable by mass for multiplexing using MS/MS.

For each new enzyme assay, individual enzyme kinetic parameters to determine optimal assay conditions will have to be established. Due to the commercial availability of exoglycosidase substrates, there might not be a unique substrate for every lysosomal enzyme in study. As a result, artificial substrates might have to be specially synthesized. Compounds used for derivatization studied earlier in this research might be useful in preparing new substrates since it has already been established that they have good MS/MS detection properties. Once a standard set of assay parameters has been determined, multiplexing could easily be accomplished by assaying all the exoglycosidases together in a single tube. When DBS is incorporated as an alternative sample type, the incubation time and amount of enzyme parameters might have to be re-evaluated, but as long as product accumulation is detectable by MS/MS, a diagnostic multiplex screening test can still be developed.

Furthermore, the β-Galactosidase and β-Hexosaminidase multiplex assay demonstrated that differing amounts of enzyme lysate and the control sample can allow varying degrees of enzyme activities or no enzyme activity to be determined through the accumulation of different levels of both 4MU and PNP detected by MS/MS. This means

that a quantification method can easily be developed using new internal standards to determine product concentrations as established earlier for mannose to $^{13}C_6$-mannose. Identifying unique products and corresponding internal standards for the group of exoglycosidases clearly illustrates the potential for assaying and quantifying enzyme activities in affected patient versus normal patient samples. By expanding this current β-Galactosidase and β-Hexosaminidase multiplex assay, a MS/MS based multiplex method for the Oligosaccharidoses could be generated to assist clinical diagnosis.

However, as mentioned before, 4-methylumbelliferyl β-D-galactopyranoside and 4-nitrophenyl N-acetyl-β-D-glucosaminide artificial substrates do not contain a natural glycosidic bond. Hence, such a multiplex assay might not be able to detect pseudodeficiencies, which are relatively common in many lysosomal hydrolases (Thomas 1994). A pseudodeficiency allele or mutation causes a protein product to be altered or changes the gene expression without causing disease. Pseudodeficiencies may result in individuals who, in spite of having greatly reduced enzyme activity against an artificial substrate determined by *in vitro* techniques carried out on fibroblasts or plasma, may remain clinically healthy due to the ability to metabolize the natural substrate *in vivo*. This will lead to a higher rate of false positive results.

The original method where disaccharides could be used as the starting substrates and monosaccharide products are followed by MS/MS to detect exoglycosidase activity is still a useful method that deserves more investigation. These starting substrates contain a true natural glycosidic linkage that may allow the assay to function more optimally. In this research, many derivatization procedures have been intensively evaluated and pure carbohydrate structures can now be easily detected by MS/MS. Since most of the target

disaccharides and corresponding internal standards are commercially available and there are a wide variety of derivatives to work with, the sequence of monosaccharide products can be further manipulated to devise a new quantitative multiplex assay for the group of exoglycosidase. Of course, enzyme kinetics will have to be re-evaluated since new substrates will be introduced. Furthermore, the concentration of endogenous monosaccharides in the fibroblasts lysate interfered with the *in vitro* products selected for detection. This situation actually might not be as significant in the other sample types such as leukocytes and DBS, which had not been examined using MS/MS as the detection technique. The UPLC-MS/MS method was also investigated briefly to try to separate isomeric carbohydrates prior to MS/MS analysis. If the right column chemistry could be determined, this might allow characterization of the types of interfering monosaccharides in the fibroblast lysates. Being able to separate monosaccharide isomers could also allow a new MS/MS detection method for more specific identification of the products of interest.

Defining a set of unique initial substrates that will generate differentiable products which can be measured by MS/MS for enzyme activity determination using either method described above will allow the goal of developing a multiplex assay to be accomplished. Enzyme kinetics is an important area that requires further analysis to better understand these enzyme reactions and to obtain more precise assay parameters. After establishing standard assay conditions, multiplexing can be tested, followed by developing quantification procedures to determine enzyme activities to diagnose these Oligosaccharidoses.

6.3. Future implementation into newborn screening program

Individual LSDs are rare, but as a group they have a prevalence similar to PKU and present a complex and challenging problem. Neonatal presentation is common and although treatment for the majority of these disorders remains unsatisfactory, developments over the next little while are likely to result in more effective therapies (Wraith 2002). Chamoles et al. had performed enzyme analysis for a number of LSDs from DBS and differentiated affected from control populations. The challenge lies in the ability to multiplex these assays to enable the screening for multiple LSDs in a single procedure. The wide availability of DBS specimens and the MS/MS technology from newborn screening laboratories supports the development of a multiplex assay. However, establishing such a screening tool requires many considerations including identification of target analytes and determination of appropriate cutoffs for analyte concentrations. Reagents, such as substrates and internal standards, must be tested and be commercially available. QC (quality control) procedures, including determination of the stability of the targeted analyte, and assay standardization, must also be developed (Marsden, Levy 2010).

The low incidence of each of these disorders makes it unlikely that screening programs for individual LSDs will be widely adopted in the near future. However, performing multiplex assays to cover a range of these disorders as currently proposed by Gelb et al. (Li et al. 2004b, Gelb et al. 2006) have been shown to be beneficial for clinical diagnosis and might one day result in their implementation into certain newborn screening programs. The continuous developments of new multiplex procedures and improved MS/MS screening methods, as described in this research for the group of lysosomal hydrolases, has already demonstrated greater advantages over the previous assays.

Conclusion

A MS/MS based multiplex assay for the detection of β-Galactosidase and β-Hexosaminidase activities has been developed through the recognition of the unique 4MU and PNP products which are released from target substrates containing particular glycosidic linkages. Earlier results demonstrating detectable enzyme activity from DBS, establishing a universal buffer, generating a quantification method using internal standards, all support that a quantitative MS/MS exoglycosidase multiplex assay can be accomplished to assist clinical diagnosis of Oligosaccharidoses. Once refined, the possible applications of this new screening tool are extensive and may include expansion to other LSDs, screening of newborn populations at risk for these genetic disorders, and assist earlier therapeutic interventions such as bone marrow, stem cell transplants, substrate reduction, gene, and enzyme replacement therapies.

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