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Molecular Analysis and Expression of the Human Glucocerebrosidase Gene

by

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B. Sc., Fujian Agricultural University, China, 1982

A Dissertation submitted in Partial Fulfillment of the Requirements for the degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming to the required standard

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Abstract

Gaucher disease is the most prevalent lysosomal lipid storage disease caused by deficient glucocerebrosidase activity. It is transmitted as an autosomal recessive trait. Three clinical forms of Gaucher disease have been described: type 1, non-neuronopathic; type 2, acute neuronopathic; and type 3, subacute neuronopathic. It has been known that in most cases, the deficient glucocerebrosidase activity is due to mutations in the glucocerebrosidase gene. However, some mutant alleles remain unidentified. In this study, we performed DNA sequence analysis of 12 mutant alleles from 6 unrelated type 1 and type 2 Gaucher patients. Two novel mutations (649T and 1366G) from one type 1 and one type 2 Gaucher patient, and two rare mutations (481T and 1604A) from two type 1 Gaucher patients were identified. To demonstrate that these mutations are deleterious and not neutral mutations, we inserted the full-length normal and mutant glucocerebrosidase cDNA into the genome of baculovirus *AcUW1.lacZ* and expressed the recombinant enzyme in *Spodoptera frugiperda* cells (*Sf9*). The levels of glucocerebrosidase activities from crude extracts of transfected *Sf9* cells with the Gaucher 649T, 1366G, 481T, and 1604A alleles are 2.8%, 2.9%, 17.3% and 6.9% of that expressed by the normal allele [normal = 352.0 nmol/hr/mg protein, using a fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (4MUGP)]. The results demonstrated that the two novel mutations (1604A and 1366G) and the two rare mutations (481T and 1604A) are deleterious, resulting in profoundly deficient glucocerebrosidase activity and subsequent Gaucher disease.

To explore the feasibility of the heterologous expression of the recombinant glucocerebrosidase in the yeast *Pichia pastoris*, we cloned the glucocerebrosidase cDNA into transformation vectors pPIC9K and pPICαZ downstream of the *AOX1* promoter, and integrated into yeast hosts KM71 and SMD1168 of *Pichia pastoris*. The recombinant glucocerebrosidase was expressed and secreted into the induced culture medium when the native targeting signal of glucocerebrosidase cDNA was replaced by an α-factor secretion signal of *Saccharomyces cerevisiae*. The maximum expression level under flask culture conditions reached the specific activity of 494 nmol/hr/mg protein on a natural substrate

(N-palmitoyl dihydroglucocerebroside). The secreted form of recombinant glucocerebrosidase was determined to have a molecular weight of 66 KDa. After deglycosylation, the peptide backbone has a molecular weight of 58 kDa. The recombinant enzyme exhibits similar kinetic properties to that of native glucocerebrosidase. A successive two-step chromatography procedure was developed to purify the recombinant enzyme to apparent homogeneity.

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List of Abbreviations

a. a.	amino acid
A	adenine
AcNPV	*Autographa californica* nucleopolyhedrovirus
AOX	Alcohol oxidase gene
APS	ammonium persulfate
Arg	arginine
Asn	asparagine
bp	base pairs
BSA	bovine serum albumin
C	cytosine
^{0}C	Celsius
CBE	conduritol B-epoxide
cDNA	complementary DNA
CTAB	hexadecytrimethyl-ammonium bromide
dATP	deoxyadenosine triphosphate
ddH_2O	distilled and deionized H_2O
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
FDA	Food and Drug Administration of the USA
FPLC	fast protein liquid chromatography
g	gram(s)
g	relative centrifugal force
G	guanine
GBA	glucocerebrosidase
His	histidine

HIS4	histidinol dehydrogenase
hr	hour
IC_{50}	inhibitor concentration that depresses enzyme activity by 50%
IgG	immunoglobulin G
kb	kilobase
kDa	kilo-Dalton
K_m	Michaelis-Menten constant
lacZ	β-galactosidase gene
Leu	leucine
M	molar
m	metre
MD	minimal dextrose
min	minute
ml	millilitre
MM	minimal methanol
moi	multiplicity of infection
mol	mole
MP	mature protein
mRNA	messenger ribonucleic acid
4MUGP	4-methylumbelliferyl-β-D-glucopyranoside
4MU	methylumbelliferyl
Mut^+	wild type for methanol utilization
Mut^s	methanol utilization slow
MW	molecular weight
n	nano (e.g. nmole = nanomole)
nt	nucleotide
NTS	native targeting signal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming units

Phe	phenylalanine
pI	isoelectric point
pKa	logarithmic scale of acid dissociation constant
pNP-Glc	paranitrophenyl β-D-glucopyranoside
Pro	proline
PT	peptide tag
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPM	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Ser	serine
Sf	*Spodoptera frugiperda*
SSCP	single strand comformation polymorphism
T	thymidine
TEMED	N, N, N', N'-tetramethylethylenediamine
U	unit
μ	micro
X-Gal	5-bromo-4-chloro-3indolyl β-D-galactoside
W	watts
V	volts
Val	valine

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Chapter 1 Introduction

1.1 Gaucher disease

Glucocerebrosidase (glucocerebroside β-glucosidase, glucosyl β–ceramidase, β-D-glucosyl-N-acylsphingosine glucohydrolase, acid β-glucosidase or EC 3.2.1.45) is a lysosomal membrane bound enzyme that hydrolyses the β-glucosidic bond of glucocerebroside. If the glucocerebrosidase activity is profoundly deficient, the natural substrate of the enzyme - glucocerebroside, will not be broken down properly. As a result, glucocerebroside will accumulate in the liver, spleen, bone marrow, and (on rare occasions) in the brain of affected individuals, causing Gaucher disease.

1.1.1 History

The first case of Gaucher disease was described in a 32-year-old female with enlargement of the liver and spleen by French physician Phillips Gaucher in 1882 (Barranger and Ginns, 1989). In 1924, Lieb characterised the storage materials accumulated in the cells of Gaucher patients as a cerebroside (Lieb, 1924). By 1965, Gaucher disease was correctly recognised as a lysosomal disorder due to a deficiency of glucocerebrosidase (Brady, 1965; Patrick, 1965). In 1966, enzyme replacement therapeutic strategy for Gaucher disease was proposed (Brady, 1966). It was demonstrated in 1974 that single intravenous infusions of purified placental glucocerebrosidase markedly reduced hepatic and blood glucocerebrosidase levels (Brady, 1974). In the early 80's, many efforts were focused on mapping the glucocerebrosidase gene on human chromosomes (Shafit-Zagardo *et al.*, 1981; Devine *et al.*, 1982; Barneveld *et al.*, 1983; Philip *et al.*, 1985). The glucocerebrosidase locus was mapped to 1q21 by somatic cell hybridization and *in situ* hybridization (Devine *et al.*, 1982; Ginns *et al.*, 1985). During the past decade, much progress has been made in

understanding the molecular biology of Gaucher disease and in the ability to treat patients with the disorder. The genomic sequence and cDNA sequence of the glucocerebrosidase gene have been delineated (Sorge *et al.*, 1985; Horowitz *et al.*, 1989). To date, nearly 110 different DNA mutations in this gene have been reported (Beutler, 1998). The procedures for diagnosis of Gaucher disease at the enzymatic level and molecular level have been well established. Treatment for Gaucher disease with enzyme replacement therapy is currently available, and research on gene therapy is under way.

1.1.2 Subtypes and clinical aspects of Gaucher disease

Gaucher disease is characterized by a remarkable degree of variability in its clinical signs and symptoms, ranging from severely affected infants to asymptomatic adults. Many patients suffer from anemia, reduced platelet counts, bone degeneration, and enlarged livers and spleens. A few develop severe central nervous system damage. Based on the presence or absence of neurologic manifestations, Gaucher disease is divided into 3 subtypes (Knudson and Kaplan, 1962). However, people with the same subtype of the disorder may differ in clinical presentation. In addition, there is no clear correlation between the level of residual enzyme activity and the severity of the different clinical subtypes (Glew *et al.*, 1988; Barranger and Ginns, 1989).

Type 1 (nonneuronopathic) is the chronic form of Gaucher disease. It may occur at any age and does not involve the nervous system. It is highly variable in both severity and progression. Clinical characteristics include anemia, thrombocytopenia, enlargement of the liver and spleen, and bone complications (Barranger and Ginns, 1989). Other organs of the monocyte/macrophage system also can be involved, including the lungs and lymph nodes (Grabowski, 1993). However, there is great variability in the organ manifestations and degree

of organ involvement among patients. Type 1 Gaucher disease is the most common form of this sphingolipidosis (Barranger *et al.*, 1989).

Type 2 (acute neuronopathic) Gaucher disease is panethnic and is a rare, rapidly progressive disorder. The early onset of neurologic involvement is characteristic of this clinical subtype. Extensive visceral involvement with hepatosplenomegaly is usually present. Most patients with type 2 disease die within the first two years of life (Frederickson and Sloan, 1978).

Type 3 (subacute neuronopathic) is characterised by a later onset of neurological symptoms, which include strasbismus, incoordination, mental deterioration, and myoclonic seizures. There is a variable degree of hepatosplenomegaly and skeletal involvement. Death occurs in early childhood. Although this type of the disease is rare and panethnic, it was found at high frequencies in certain population isolates in northern Sweden (Erikson, 1986).

A consistent feature of Gaucher disease is the presence of Gaucher cells (lipid-laden macrophages), as a result of glucocerebroside accumulation in the lysosomes (Parkin and Brunning, 1982). These Gaucher cells are large (20 to 100 µm), and have an eccentric nucleus, and a "wrinkled tissue paper" appearance to the cytoplasm (Parkin and Brunning, 1982). Most of the storage material within Gaucher cells is derived from phagocytosis of cells, cell membranes, and cell debris that are external to the cell and are not from cell-specific glycolipid synthesis (Beutler and Grabowski, 1995). Gaucher cells are abundant in the spleen, liver, lymph nodes, and bone marrow. However, the accumulation of glucocerebroside can occur in any tissues, including the central nervous system (Beutler and Grabowski, 1995).

1.1.3 Population genetics

Gaucher disease is the most common lysosomal storage disease. Estimates of the disease frequency for type 1 Gaucher disease ranges between 1/60,000 and 1/40,000 in the general population (Grabowski, 1993). Although it is panethnic, type 1 Gaucher disease occurs more frequently in individuals of Eastern European (Ashkenazi) Jewish descent, making it the most prevalent Jewish genetic disorder. The incidence among this group is estimated to between 1 in 2500 to 1 in 600 (Kolodny *et al.*, 1979, Matoth *et al.*, 1987). Type 2 and type 3 are much more rare than type 1. The estimated disease frequency of type 2 is less than 1 in 100,000 live births, and that of type 3 is between 1/100,000 to 1/50,000 live births (Grabowski, 1993).

1.2 Glucocerebrosidase and the natural substrate of the enzyme

1.2.1 Biochemistry and molecular biology of glucocerebrosidase

Glucocerebrosidase has catalytic activity for the hydrolysis of the β-glycosidic bond of glucocerebroside. The protein is tightly membrane-bound (Imai, 1985). Detergents are required to solublize the enzyme from membranes. The protein is composed of 497 amino acids, with a calculated molecular weight of 55,575 Da (Erickson *et al.*, 1985). About 13% of the residues are basic (lysine, arginine or histidine) and the calculated pI value is 7.2 (Beutler and Grabowski, 1995).

1.2.1.1 Biosynthesis

The human glucocerebrosidase is initially synthesised as a precursor polypeptide with a signal sequence in its N-terminus in the endoplasmic reticulum (Erickson *et al.*, 1985; Jonsson *et al.*, 1987). The polypeptide is glycosylated cotranslationally. Glycosylation is required for the catalytic activity of glucocerebrosidase (Grace and Grabowski, 1990). Based

on data from isoelectric focusing, and Western blotting of cell and tissue extracts, the enzyme is extensively posttranslationally processed. Three forms of glucocerebrosidase from human fibroblast were observed (Ginns *et al.*, 1982). The early peptides, containing "high mannose" oligosaccharides, are sensitive to endoglycosidase H digestion. The final glycosylated form of the enzyme contains four oligosaccharide chains (Erickson *et al.*, 1985). The majority of the oligosaccharide side chains are typical biantennary and triantennary complex-types that contain sialic acid, galactose, N-acetylglucosamine, mannose, and fucose (Takasaki *et al.*, 1984). Out of five potential glycosylation sites on the glucocerebrosidase structural peptide, four are being utilized (Takasaki *et al.*, 1984). Unlike other lysosomal enzymes, glucocerebrosidase does not undergo oligosaccharide phosphorylation in the Golgi apparatus, a process for targeting soluble lysosomal enzymes to the lysosomes of cells (Aerts *et al.*, 1988).

1.2.1.2 Isolation and purification

Glucocerebrosidase was first isolated from human placenta using cholate extraction (Furbish *et al.*, 1977). The enzyme was purified to homogeneity with hydrophobic interaction and gel-permeation chromatography (Furbish *et al.*, 1977; Murray *et al.*, 1985; Choy, 1986). Active human glucocerebrosidase has also being isolated using either substrate analog affinity or monoclonal antibody affinity columns (Barneveld *et al.*, 1983; Grabowski and Dagan, 1984; Osiecki-Newman *et al.*, 1986).

1.2.1.3 Stimulators, activator proteins and inhibitors

Bile salts, negatively charged phospholipids, and negatively charged detergents (taurocholate) stimulate the activity of glucocerebrosidase *in vitro* (Dale *et al.*, 1976; Mueller and Rosenberg, 1979; Grabowski *et al.*, 1982). Thoses have been useful in diagnostic procedures as a mean of stimulating glucocerebrosidase activity and inhibiting interfering β-

glucosidases, thus improving the sensitivity and specificity of tests for glucocerebrosidase activity (Baranger and Ginns, 1989).

An activator protein called factor P was reported by Ho and O'Rrien (1971). The factor P was also called saposin C or sphingolipid activator protein-2 (SAP-2), but saposin C is now the term in common usage (Grabowski, 1993). Molecular cloning of activator protein cDNA revealed that four sphingolipid hydrolase activator proteins are encoded by a single gene (prosaposin) (O'Brien *et al.,* 1988; Gavrieli-Rorman and Grabowski, 1989). Two of those activator proteins (saposin A and C) have been isolated from human (Morimoto *et al.,* 1989; Kleinschmidt *et al.,* 1989), and guinea pig (Sano *et al.,* 1988b). Saposin C is a very acidic and heat stable glycoprotein, which is composed of 80 amino acid residues (Sano and Radin, 1988a; Sano *et al.,* 1988b). The physiological roles of this activator protein are not yet fully understood. However, the use of inhibitory monoclonal antibodies has provided direct support for saposin C causing a conformational change in glucocerebrosidase that enhances catalytic activity (Paton and Poulos, 1988; Fabbro and Grabowski, 1991). Several reports revealed that saposin C deficiency can cause Gaucher disease (Harzer *et al.,* 1989; Christomanou *et al.,* 1989; Rafi *et al.,* 1993).

Several inhibitors have been found to inhibit the activity of human glucocerebrosidase. 1,5-D-gluconolactone and 1-deoxy-nojirimycin are reversible inhibitors of glucocerebrosidase (Osiecki-Newman *et al.,* 1987; Baranger and Ginns, 1989). Conduritol B-epoxide (CBE) is an irreversible inactivating inhibitor (Daniels *et al.,* 1980; Lee *et al.,* 1985).

1.2.2 The natural substrate of glucocerebrosidase

Glucocerebroside (N-acyl-sphingosyl-1-*O*-β-D-glucoside, ceramide β-glucoside or glucosylceramide) is a glycolipid composed of ceramide and glucose (Figure 1.1). The glucose

moiety is attached to the C-1 of ceramide in a β-glucosidic linkage. Ceramide is derived from the long-chain base named sphingosine [D(+)-erythro-1,3-dihydroxy-2-amino-4-transoctadecene, or C_{18} sphingosine] (Barranger and Ginns, 1989). This lipid is joined by an amide bond at C-2 to a long-chain fatty acid to form ceramide. The length of fatty acid chains varied from C_{18} to C_{24} (Nilsson *et al.,* 1985). Glucocerebroside is widely distributed in many mammalian tissues in small quantities as a metabolic intermediate in the synthesis and degradation of complex glycosphingolipids and as a membrane constituent (Grabowski, 1993). Glycosphingolipids and gangliosides are broken down in a stepwise fashion by specific acid hydrolases, resulting in the formation of glucocerebroside. Glucocerebroside is then normally degraded to ceramide and glucose by glucocerebrosidase (Barranger, J.A. *et al.,* 1989). The amount of glucocerebroside in the plasma, liver, spleen and/or brain of Gaucher disease patients is elevated (Beutler and Grabowski, 1995).

1.3 Glucocerebrosidase gene and mutations

1.3.1 Glucocerebrosidase gene

The clones containing the human glucocerebrosidase cDNA were isolated using λgt11 expression libraries, and the complete nucleotide sequence of the cDNA was determined (Sorge, *et al.,* 1985; Tsuji *et al.,* 1986). The full length glucocerebrosidase cDNA is about 1.7 kb. The sequences of the entire functional gene and a pseudogene have also been determined (Horowitz *et al.,* 1989). Located on chromosome 1q21, the human glucocerebrosidase functional gene contains 11 exons and 10 introns with a total length of 7 kb. The human glucocerebrosidase cDNA was found to contain two in-frame ATG start codons in its long open reading frame (Sorge *et al.,* 1987). Up-stream of the amino terminus of the protein, there are sequences coding for a signal peptide and a glycine peptidase

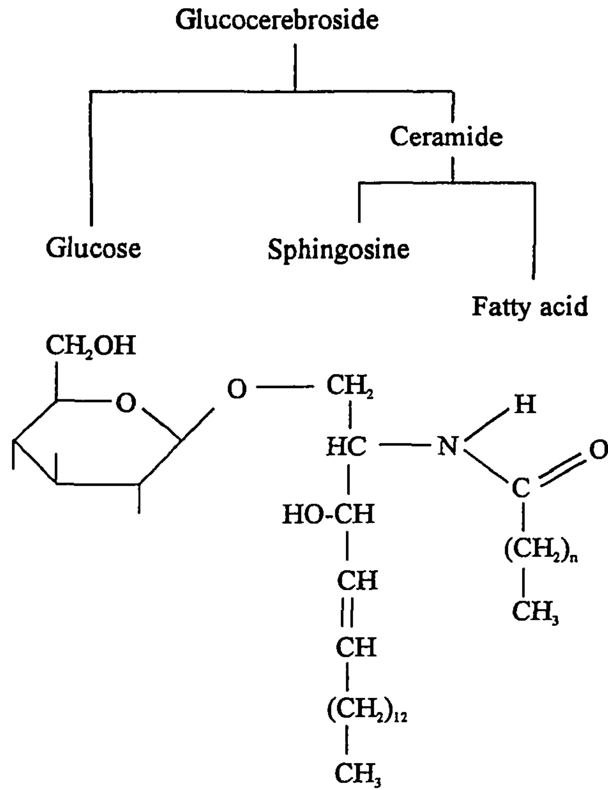


Figure 1.1 Glucocerebroside: natural substrate of glucocerebrosidase. This figure is modified from Principles of Biochemistry (Horton *et al.*, 1992).

cleavage site (Ginns *et al.*, 1985). The sequences of the signal peptide can be 39 or 19 amino acids depending which one of the two start codons is used. The signal peptides that are initiated from each ATG differ in their hydrophobicity (Sorge *et al.*, 1987). It was found that either ATG could function as the initiation codon for glucocerebrosidase synthesis in cultured fibroblasts and target the enzyme to the lysosomes (Sorge *et al.*, 1987). The mouse cDNA has been cloned and appears to have only a single start codon, which is homologous to the human downstream start codon (O'Neil *et al.*, 1989).

1.3.2 Glucocerebrosidase pseudogene

A pseudogene of glucocerebrosidase was found to be located about 16 kb downstream from the functional glucocerebrosidase gene (Horowitz *et al.*, 1989; Zimran *et al.*, 1990). The pseudogene is highly similar to the functional gene with 96% identity in the regions present in both sequences. However, certain portions of the functional gene are not represented in introns 2, 4, 6, and 7, and at several splice junctions of the pseudogene. There is a 55 bp deletion from the coding region of exon 9, as well as a 5 bp deletion from the coding region of exon 4. There are also base pair changes scattered throughout the pseudogene (Horowitz *et al.*, 1989).

In their examination of cellular RNA by RT-PCR using lymphoblasts or fibroblasts from Gaucher patients and normal subjects, Sorge *et al.* (1990) found that the pseudogene was consistently transcribed. They also found that, in some cases, the level of transcription appeared to be approximately equal to that of the functional gene. Reiner and Horowitz (1988) found that the promoter of the glucocerebrosidase pseudogene has demonstrable activity when attached to a reporter gene, but much less than that of the functional gene. They commented that mutations in the rest of the gene must render the mRNA vulnerable

to breakdown or other functional abnormality such that no enzyme is synthesized. However, Imai *et al.* (1993) reported a novel transcript from a pseudogene in non-Gaucher disease cells, and *in vitro* translation of a polypeptide appeared to be approximately 30 kDa. It is speculated the pseudogene was formed by random duplication during evolution (Beutler, 1995c). PCR amplification of the functional gene for mutation analysis can be complicated by the presence of this highly similar pseudogene. Tayebi *et al.* (1996) reported a method to distinguish the glucocerebrosidase gene from the pseudogene, involving the use of long PCR to simultaneously generate a 5.6 kb fragment from the functional glucocerebrosidase gene and a 3.9 kb fragment from the pseudogene.

1.3.3 Mutations of the glucocerebrosidase gene

To date, nearly 110 glucocerebrosidase gene mutations have been described in patients with Gaucher disease (Beutler and Gelbart, 1998). The types of mutations include missense mutations, frameshift, and splicing mutations, as well as deletions, insertions and recombination with the pseudogene. In a model created by introducing a null allele in embroynic stem cells through gene targeting, homozygous mutant mice were produced which were profoundly deficient in glucocerebrosidase activity. They died within twenty-four hours of birth (Tybulewicz *et al.*, 1992). This demonstrated that knockout mutations can have a dramatic impact on the viability of the organism. Based on the observed phenotypic effects, Beutler and Gelbart (1998) divided the mutations into three groups: null alleles, severe alleles, and mild alleles. Null alleles do not direct any enzyme production. Severe alleles are those, when inherited with a null or another severe allele, usually associated with neuronopathic disease (type 2 and type 3). Mild alleles are those that are associated with non-neuronopathic disease (Beutler *et al.*, 1994; Beutler and Gelbart, 1998).

Because the gene frequency of type 1 Gaucher disease is elevated in Jewish population, mutation detection in the 1980's was mainly focused on Gaucher patients among this population. Four most frequent mutations constituted about 96% of the total mutations in the Ashkenazi Jewish population have been described (Buetler *et al.,* 1993b). The first mutation is an A to C transition in cDNA 1226 (genomic DNA 5841). It results in asparagine to serine substitution at amino acid position 370. It was found to be associated with a mild clinical phenotype (Tsuji *et al.,* 1988; Zimran *et al.,* 1989). This mutation accounts for 77% of disease-producing alleles in the Jewish population (Zimran *et al.,* 1991; Buetler, 1992a). The second mutation is a T to C transition in cDNA nt 1448 (genomic DNA 6433), which was first reported by Tsuji *et al.,* (1987) in a patient with type 2 Gaucher disease. This mutation results in leucine to proline substitution at amino acid position 444 of glucocerebrosidase. It accounts for about 3% of the mutant alleles in the Jewish Gaucher patients (Buetler *et al.,* 1991a). The third mutation is an insertion of an extra G at nt 84 (84GG), which accounts for an additional 13% of the mutations in Jewish population (Beutler, 1992b). The forth most frequent mutation is at the upstream splice junction of intron 2 (IVS2+1), representing about 3% of the total mutations of glucocerebrosidase in Jewish Gaucher patients (Beutler *et al.,* 1992a).

Glucocerebrosidase gene mutations of non-Jewish populations were found to have a wider spectrum. The four most frequent mutations among the Jewish population only account for 75% of the mutations among the non-Jewish population (Beutler and Gelbart, 1993a). It was noted that mutation 1448C is elevated among non-Jewish patients. It accounts for 37% and more than 40% of the Gaucher disease-producing alleles in British/Irish and Japanese Gaucher patients respectively (Hatton *et al.,* 1997; Ida *et al.,* 1997). Mutation 1448C was also

found to be common among Korean and Chinese Gaucher patients. It accounts for 50% of Korean Gaucher disease-producing alleles and 40% of Chinese Gaucher disease-producing alleles (Kim 1996; Choy *et al.,* 1997). Mutation 1226G accounts for 18% of the Gaucher disease-producing alleles in British and Irish Gaucher patients (Hatton *et al.,* 1997). This mutation was not found among 96 Japanese, 5 Korean, and 5 Chinese Gaucher patients tested (Ida *et al.,* 1995; 1996; 1997; Kim *et al.,* 1996; Choy *et al.,* 1997).

The presence of the glucocerebrosidase pseudogene complicates the amplification of the functional gene *in vitro.* If DNA recombination occurs between functional gene and pseudogene, it can also cause deleterious effects on the functional gene *in vivo.* Multiple mutations in the functional gene appear to have arisen as recombination events with the pseudogene (Eyal *et al.,* 1990; Hong *et al.,* 1990; Latham *et al.,* 1990, 1991; Zimran *et al.,* 1994; Strasberg *et al.,* 1994; Hatton *et al.,* 1997). Zimran and his co-workers (1989) detected a mutation which represented crossing-over between the glucocerebrosidase gene and the pseudogene, resulting in a fusion gene designated XOVR. Another glucocerebrosidase fusion gene that consisted of the 5' end of the functional gene and the 3' end of the pseudogene was also reported by these investigators (Zimran *et al.,* 1990).

1.3.4 Molecular aspects of Gaucher mutations

Mutations in the glucocerebrosidase gene may affect the enzyme properties in various respects. Although normal amounts of mRNAs were found to be present in fibroblast extracts from several subtypes of Gaucher disease by Northern blotting analysis (Graves *et al.,* 1986), it was speculated that some mutations affect the stability of the mRNA transcripts. Mutations can result in different kinetics properties of the enzyme. Several reports revealed that different kinetic properties of the residual enzyme were present in

patients with different subtypes of Gaucher disease (Klibansky et al., 1973; Choy and Davidson, 1980). It was also noticed that the mutant protein is somewhat unstable (Bergmann and Grobowski, 1989). Beutler *et al.* (1984) studied the processing of glucocerebrosidase in fibroblast of different subtypes Gaucher disease by using biosynthetic labeling and immunoprecipitation. In normal fibroblasts, a 60 kDa glucocerebrosidase polypeptide antigen was initially present. It was gradually replaced by a 63 kDa glucocerebrosidase polypeptide antigen. It was presumed that the 63 kDa band is a mature enzyme. Processing of glucocerebrosidase in six unrelated patients with type 1 and in one patient with type 3 was the same as that of the normal. In contrast, 3 patients with the severe type 2 form showed an unstable enzyme. The 60 kDa band appeared only transiently and the mature 63 kDa band was never seen (Beutler *et al.*, 1984). Thus, an unstable precursor characterizes type 2 Gaucher disease. In other cases of Gaucher disease, the mutations seem to preclude localization of the mutant glucocerebrosidase to the lysosomes (Willemsen *et al.*, 1988).

1.4 Correlation of genotypes and phenotypes

Mutation analysis provides precise diagnosis but may not give information concerning the severity or progression of the disease. Much effort has been focused on the correlation of genotypes and phenotypes. To date, except for some mutations that cause early termination of protein translation, only a few mutations have been well characterized, and the correlation of those mutations to phenotypes is well documented. Mutation 1226G and 1448C are two examples. Mutation 1226C in Gaucher patients does not lead to neuronopathic involvement. It is frequently found in either the heteroallelic and homoallelic form among Jewish and non-Jewish type 1 Gaucher patients, but is absent in all type 2 and 3

Gaucher patients surveyed (Sibille *et al.,* 1993). Some patients homozygous for mutation 1226G are entirely symptom-free, and glucocerebrosidase deficiency has not affected their health. The clinical features of the 1226G homozygotes were usually related to splenomegaly and thrombocytopenia in patients who had symptoms (Zimran *et al.,* 1989). The presence of mutation 1226G might prevent the development of central nervous system abnormalities (Tsuji *et al.,* 1988). Homozygous mutation 1448C among Gaucher patients generally presents with neuronopathic type 2 and type 3 disease of variable severity (Zimran *et al.,* 1989).

Although some generalizations can be made about mutations that are more frequently encountered in particular patient populations, Gaucher patients sharing identical genotypes can exhibit considerable clinical heterogeneity (Sidransky and Ginns, 1993). It has also been noted that other genetic and non-genetic factors, such as environmental factors, may be involved in the expression of the disease (Beutler, 1991b; Beutler *et al.,* 1996). In the Norrbottnian population of Sweden, with a homogeneous genetic mutation (1448C) at the glucocerebrosidase locus, there is extensive phenotypic heterogeneity (Svennerholm *et al.*, 1982; Dreborg *et al.,* 1980; Erikson *et al.,* 1987). In Japanese population, homozygous mutation 1448C is associated with non-neuronopathic disease, indicating that genotype/phenotype may vary with genetic background (Grabowski, 1993; Ida *et al.,* 1996). For most of the mutations identified, it is still not clear to what extent a person's clinical features (phenotype) or prognosis can be accurately predicted through mutation analysis.

1.5 Diagnosis and treatment of Gaucher disease

1.5.1 Diagnosis of Gaucher disease

Although the presence of Gaucher cells within the tissues of the Gaucher patients is a consistent feature of the disease, histochemical diagnosis of Gaucher disease is not widely used. This is because it requires an invasive procedure, and the presence of similar cells, pseudo-Gaucher cells, in a variety of other disorders (Grabowski, 1993; Beutler, 1992b). The determination of leukocyte or fibroblast glucocerebrosidase activity makes possible a relatively simple laboratory diagnosis of Gaucher disease. However, there is no clear correlation between the level of residual glucocerebrosidase and the clinical severity associated with the different forms of the disease (Barranger *et al.*, 1989).

Although a variety of natural and artificial substrates provide accurate assays, all have similar limitations when used for heterozygote detection. To establish 95% confidence ranges for inclusion of all obligate heterozygotes, about 25-30% of normal controls will be in the heterozygote range (Grabowski, 1993). The artificial substrate 4-methylumbelliferyl-β-D-glucopyrannoside offers a relatively simple method for the enzymatic assay of glucocerebrosidase. For a more accurate diagnosis, the natural substrates, such as N-palmitoyl-DL-dihydro-glucocerebroside, should be used for the assay. Conduritol B-epoxide is a specific inhibitor of mammalian glucocerebrosidase. It permits the confirmation of the enzyme deficiency in a system where non-specific β-glucosidase may be interfering (Radin and Bernet, 1982).

The instability of leukocyte glucocerebrosidase and the overlap in activities observed in normal subjects and heterozygotes limits the usefulness of enzyme based diagnostics under some circumstances (Beutler, 1992b). DNA-based molecular

identification of mutations in the glucocerebrosidase gene can provide accurate mutation screening and carrier detection, particularly in defined populations. Since DNA is very stable, much information can be retrieved from biopsies even when patients are deceased. Currently, the methods for diagnosis by DNA analysis include the PCR amplification of genomic DNA fragments, RT-PCR of cDNA, SSCP, RLFP and DNA sequence analysis.

1.5.2 Symptomatic measures and organ transplantation

As a genetic disorder, Gaucher disease can be treated at various levels. All of the various treatments have been useful in prolonging and improving the life quality of Gaucher patients. Surgical splenectomy and bone marrow transplantation have been reported in reversing symptoms of several type 1 Gaucher disease patients (Barranger, *et al.*, 1989). Enzyme replacement therapy is now being implemented, and the research on gene therapy is underway (Beutler and Grabowski, 1995).

Gaucher disease has been traditionally managed by supportive therapy including total or partial removal of the spleen, blood transfusions, orthopedic procedures, and occasionally bone marrow transplantation (Barranger and Ginns, 1989). Some type 1 Gaucher patients require splenectomy for management of thrombocytopenia and anemia (Fleshner *et al.*, 1991) However, splenectomy is often followed by an increase in bone involvement, with osteolytic lesions within a few months of surgery (Ashkenazi *et al.*, 1986). Partial splenectomy has been advocated with the dual goals of minimizing the deleterious effect on bone and avoiding postsplenectomy sepsis (Rubin *et al.*, 1986).

Several attempts have also been undertaken to provide the patient with tissue capable of the continuous release of active enzyme. Renal transplantation has had little effect on the disease process in patients with Gaucher disease (Desnick, 1973). Starzl *et*

al. (1993) reported that liver transplantation in a patient with type 1 Gaucher disease resulted in dramatic reduction in the lymph-node deposits of glucocerebroside.

Gaucher disease can be cured by bone marrow transplantation (Grabowski, 1993). Although bone marrow transplantation has been used successfully in the past to treat a few people with Gaucher disease, the treatment requires a marrow donor and has a 10 to 25 percent risk of fatal complications. This makes it a less desirable treatment for most Gaucher patients (Parkman, 1986). Nevertheless, bone marrow transplantation may be an appropriate treatment method for type 3 disease, since it is unknown if the neurologic disease could be prevented or arrested by the administration of glucocerebrosidase, which does not cross the blood-brain barrier (Beutler and Grabowski, 1995).

1.5.3 Enzyme replacement

During the past decade, progress has been made in enzyme replacement treatment for Gaucher disease. Initial research on the natural glucocerebrosidase enzyme by Brady *et al.* (1974, 1975) showed that it was not particularly effective when administered by infusion to people with Gaucher disease. The majority of the enzyme did not reach the "Gaucher cells" in the body, presumably because most of the enzyme was taken up by hepatocytes. Based on the discovery of a specific receptor for α-mannosyl-terminated oligosaccharides on macrophages (Ashwell and Morell, 1974; Achord *et al.*, 1978; Stahl *et al.*, 1978), a strategy that modified the carbohydrate side chains of glucocerebrosidase to increase targeting and uptake in the macrophages was developed (Furbish *et al.*, 1978; Doebber *et al.*, 1982; Barton *et al.*, 1990). The modified enzyme has terminal mannose sugars that are specifically bound by a protein on the macrophage plasma membrane. Once bound, the enzyme is internalized and delivered to the lysosome (Barton *et al.*, 1991).

Modified glucocerebrosidase has been evaluated in several other clinical trials in type 1 disease (Beutler and Grabowski, 1995).

There is good evidence that enzyme replacement therapy with placental or recombinant glucocerebrosidase is beneficial for type 1 Gaucher patients in reversing many of the manifestations of the disorder, including abnormal blood counts, increased liver and spleen size, and some skeletal abnormalities (Beutler *et al.,* 1991; Kay *et al.,* 1991; Barton *et al.,* 1991). Enzyme therapy appears to obviate the need for splenectomy in most cases. Several patients with type 2 disease have been treated with enzyme replacement therapy, without substantial improvement in their neurologic problems (Erikson *et al.,* 1993; Balicki and Beutler, 1995). With current technology, enzyme replacement therapy is unlikely to be effective for patients with type 2 disease. The efficacy of enzyme replacement for neurologic abnormalities in type 3 disease remains to be established (Bembi *et al.*, 1994; Zimran *et al.*, 1995).

Currently, there are two types of glucocerebrosidase available commercially. Ceredase (Alglucerase for injection), a mannose-terminated placental glucocerebrosidase produced by Genzyme Corporation of Cambridge, Massachusetts, was approved by the US Food and Drug Administration (FDA) in 1991. In mid-1994, the FDA approved a recombinant alternative to Ceredase, Cerezyme (Imiglucerase for injection), also produced by Genzyme (Fox, 1995). Patients receive the modified enzyme by intravenous infusion. The usual recommended dose is 60 units per kilogram of body weight every 2 weeks for 9 to 12 months (Barton *et al.,* 1990). The enzyme unit (U) is defined as the amount of enzyme that catalyzes the hydrolysis of one μmole of the synthetic substrate paranitrophenyl β-D-glucopyranoside (pNP-Glc) per minute at 37 ^{0}C (Genzyme Corp.

Boston, MA). There is a continuing controversy over aspects of enzyme replacement therapy for individuals with type 1 disease, such as dosage, methods, frequency of administration, and cost. The most contentious issue, and potentially the most difficult for patients and their physicians, is enzyme dosage. Clinical successes have been observed with both the "high" (120 U/kg/4 weeks) and "low" (30 U/kg/4 weeks) dosage regimens (described as amount of enzyme administered during a 4-week interval for purposes of comparison, independent of dosage schedule) (Mistry *et al.*, 1992; Figueroa *et al.*, 1992; Pastores *et al.*, 1993, Zimmran *et al.*, 1993, 1995).

Enzyme replacement therapy for Gaucher disease patients seems to be quite safe, with only a few adverse reactions being reported, including chills, fever, and gastrointestinal disturbances (Grabowski, 1993). As with any protein therapy, there is a possibility that patients may develop an immune reaction (antibodies) to the infused enzyme. Antibody analysis has shown the antibody formation rate to be approximately 10 - 15% of cases after enzyme replacement treatment (Richards *et al.*, 1993; Beutler, 1995).

Despite its promise, the great drawback with enzyme replacement therapy is that it involves lifelong infusions and the cost of the treatment. The standard regimen of Ceredase can cost $140,000 - 350,000 US dollars annually per adult patient (Garber, 1992). An alternative, more cost-effective treatment or means of producing the enzyme glucocerebrosidase in mass quantities is desirable. With the development of a cost effective recombinant enzyme preparation, it is hoped that the cost of treatment will decline in the near future, allowing this method to be available to a greater number of Gaucher patients.

1.5.4 Gene therapy

Recombinant DNA technology has made it possible to consider the correction of genetic disease at the most fundamental level, the gene. As a recessive single gene disorder, Gaucher disease is an excellent candidate for gene therapy. By transferring functional copies of glucocerebrosidase cDNA to the patient, permanent correction of the reversible features of the mutant phenotype may be possible. From the results of bone marrow transplantation and enzyme replacement therapy, we know that a supply of glucocerebrosidase reverses disease symptoms and stops the progression of the disease. Therefore, corrected hematopoietic stem cells that take up residence in the bone marrow may provide a permanent source of glucocerebrosidase-producing macrophages (Barranger and Ginns, 1989).

The interests in gene therapy for Gaucher disease have led to the development of several protocols for glucocerebrosidase cDNA transfer. Retroviral vectors have been a major focus as efficient vehicles for the transfer of the glucocerebrosidase cDNA into various host cell lines (Kohn *et al.*, 1989; Barranger, 1993; Balicki and Beutler, 1995). Human glucocerebrosidase cDNA transferred into mouse fibroblasts was readily distinguished from the mouse enzyme using mouse monoclonal anti-glucocerebrosidase antibodies (Sorge *et al.*, 1987). Cultured fibroblasts from a patient with type 1 Gaucher disease were transfected with the retrovirus containing glucocerebrosidase cDNA. The level of enzyme activity was restored to normal, while uninfected cells or cells infected with virus only did not produce glucocerebrosidase (Choudary *et al.*, 1986). In another report, type 2 Gaucher cells infected with a retrovirus carrying the human cDNA were corrected to normal levels of glucocerebrosidase activity (Barranger *et al.*, 1989). It has

also been demonstrated that retroviral vectors can efficiently transfer the glucocerebrosidase gene into long-lived hematopoietic progenitor cells from the bone marrow of Gaucher disease patients and express physiologically relevant levels of enzyme activity (Nolta *et al.*, 1992; Xu *et al.*, 1994). An adeno-associated virus vector was also constructed for the expression of the human glucocerebrosidase in patient fibroblasts (Wei *et al.*, 1994).

Some other risks associated with this clinical trial are related to the retroviral vector. The healthy copy of the glucocerebrosidase gene may be inserted into an undesirable location in the stem cell DNA, where it might interfere with an essential gene or activate an undesirable gene (*i.e.*, an oncogene). If this occurred, it could cause cells to grow in an abnormal way and possibly lead to cancer or leukemia. Another concern is that the retrovirus used as the vector may regain the ability to replicate itself and multiply (*i.e.*, through recombination with the wildtype). A replication-competent retrovirus may cause infection and might increase the chance of a patient developing cancer or leukemia. It is hoped that a better understanding of the *in vivo* regulation of expression following gene transfer will help bring treatment of Gaucher disease closer to fruition. Although obstacles remain to be overcome, Gaucher disease may be the first lysosomal storage disease to be treated using gene therapy. This success would provide hope for similar treatment of other inherited lysosomal storage disorders.

1.6 Thesis objective

1. To identify unknown mutations among various fibroblast cell lines or DNA samples from patients with different clinical forms of Gaucher disease. More than 60% of the mutant alleles that have been enzymatically diagnosed previously in our collection

remain unidentified at the DNA level. Those mutant alleles will be screened for the presence of mutations using PCR amplification and RFLP analysis. If the results are negative, sequence analysis will be performed to identify the unknown mutations. The data obtained will be useful in understanding the molecular basis of Gaucher disease.

2. To characterize various novel missense mutations identified by expressing the mutant and normal alleles of the glucocerebrosidase using the baculovirus expression system in insect cells. It is essential to demonstrate if novel mutations identified are causative for Gaucher disease. The mutant and normal glucocerebrosidase cDNA will be recombined into the baculovirus genome and expressed in transfected insect cells. The mutant enzyme will be characterized using enzyme activity analysis. The *in vitro* enzyme activity analysis of the expressed mutant enzyme may have an implication in the correlation of genotype and phenotype in the patients with Gaucher disease.

3. To explore the feasibility of heterologous expression of recombinant human glucocerebrosidase in the yeast *Pichia pastoris*. As an eukaryotic microorganism, *Pichia pastoris* has several advantages such as cost-efficiency, post-translational modification, and high levels of expression. The functional human glucocerebrosidase cDNA will be integrated into the *Pichia* genome, and recombinant enzyme-producing clones will be selected. The recombinant enzyme will be purified and characterized. Studies of cloning, expression, characterization and purification of the recombinant glucocerebrosidase from *Pichia pastoris* will provide information for eventual mass scale production of the enzyme for potential therapeutic purposes.

Chapter 2 Molecular Analysis of Mutant Glucocerebrosidase Alleles

2.1 Abstract

Gaucher disease is the most prevalent lysosomal lipid storage disease caused by deficient glucocerebrosidase activity. It is transmitted as an autosomal recessive trait. Three clinical forms of Gaucher disease have been described: type 1, non-neuronopathic; type 2, acute neuronopathic; and type 3, subacute neuronopathic. The principal difference between the subtypes is the presence and progression of neurologic complications. In most cases, the deficient glucocerebrosidase activity is due to mutations in the glucocerebrosidase gene. However, some mutant alleles remain unidentified. In this study, we performed DNA sequence analysis of 12 mutant alleles from 6 unrelated type 1 and type 2 Gaucher patients in North America. A method of direct sequence analysis of full-length glucocerebrosidase cDNA without cloning was developed. Two novel mutations (649T and 1366G) from one type 1 and one type 2 Gaucher patients, and two rare mutations (481T and 1604A) from two type 1 Gaucher patients were identified. Mutation 649T results in Pro to Ser substitution at amino acid residue 178. Mutation 1366G results in Asn to Ser substitution at amino acid residue 417. Methods for clinical diagnostic applications in the identification of those mutations were also developed (Choy *et al.*, 1994a, 1994b, 1995).

2.2 Introduction

Gaucher mutations within the non-Jewish population exhibit a heterogeneous distribution (Amaral *et al.*, 1993; Walley *et al.*, 1993; Cormand *et al.*, 1995; Ida *et al.*, 1995; Coutre *et al.*, 1997). In addition, more than 25 % of the total mutations among non-Jewish Gaucher patients remain unidentified (Beutler *et al.*, 1990, 1992; Horowitz *et al.*,

1993). Our efforts towards the identification of Gaucher mutations have thus focused on disease alleles from non-Jewish patients. In this study, fibroblast cell lines from six unrelated Gaucher patients in North America, with type 1 and type 2 clinic subtypes, were collected and subjected to genomic DNA or cDNA sequence analysis. We have reported the identification of two novel mutations (649T and 1366G) from one type 2 and one type 1 Gaucher patients, and two rare mutations (481T and 1604A) from two type 1 Gaucher patients. Simple diagnostic methods which utilize the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis for the identifications of these mutations are described (Choy *et al.*, 1994a, 1994b, 1995).

2.3 Patients

Patient BL is a non-Jewish Caucasian type 1 Gaucher patient. She is of French-Finnish and French-Ukrainian descent. She was investigated at age 6 1/2 years for unexplained splenomegaly. There was no history of bone pain or neurological problems. The diagnosis of Gaucher disease was raised and confirmed by the presence of Gaucher cells in bone marrow aspirate. Fibroblast culture followed by glucocerebrosidase activity assay confirmed the clinical diagnosis of Gaucher disease. She was readmitted at age 13 1/2 years because of hypersplenism. The spleen was large and hard, filling the whole left abdomen. She underwent a splenectomy that reversed her hematological complications. At the age of 21 years, she was asymptomatic and there was no clinical evidence of hepatic or neurological involvement (Choy *et al.*, 1994b).

Patient BD was an African-American patient who died at age 1 with type 2 Gaucher disease. He was found to be heterozygous for mutation 1448C. The second allele has an unknown mutation (Choy *et al.*, 1991). The fibroblast cell line was provided

by Dr. Yoav Ben-Yoseph at Wayne State University Medical Center (Choy and Wei, 1995).

Patient ES was initially diagnosed at age 23 years. He is the second child born to a nonconsanguineous Ashkenazi Jewish couple. The father's family came from Russia and the mother's family came from Poland and Austria. At age 23, routine physical examination disclosed splenomegaly. A bone marrow examination demonstrated megaloblastic anemia and the presence of Gaucher cells. He had no previous problems with anemia, bleeding disorders or bone pain. A diagnosis of Gaucher disease was made on the basis of the bone marrow results and confirmed by assay of β-glucosidase activity in cultured skin fibroblasts. At splenectomy, the splenic parenchyma was noted to have been replaced by masses of large, pale-staining reticuloendothelial cells. The patient was then well until age 33 years when he experienced a sudden onset of severe pain involving the upper thigh. Hip radiography demonstrated "nondescript" lytic lesions of the head of the femur but these were not radiologically typical of Gaucher disease. By age 40, a hip replacement was done because of continuing severe pain. The other hip continues to have mild pain. At age 43, he was found to be hypertensive and investigation documented bilateral glomerulosclerosis. During the biopsy procedures, a unilateral renal cell carcinoma was unexpectantly found and a partial nephrectomy was performed. By age 46, he has no significant anemia or bleeding disorder. The patient is neurologically completely unimpaired. He is of high intelligence and has no neurological problems (Choy *et al.*, 1994a).

Patient JB is a juvenile native Indian patient with type 1 Gaucher disease. The fibroblast cell line was sent to us by Dr. Patrick Ferreira in Alberta. The patient has developmental delay, hematological complications and bone crisis.

Patient CR is a type 1 Gaucher patient. The fibroblast cell line was sent to us by Dr. Fiona Bamforth at the University of Alberta Hospitals.

Patient VD is a non-Jewish European. She was diagnosed at age of 20 as having type 1 Gaucher disease. The fibroblast cell line was sent to us by Dr. C. Clark at the Kinston General Hospital, Ontario.

2.4 Materials and methods

2.4.1 Materials

The following were from commercial sources: RPMI 1640, fetal bovine serum, trypsin, penicillin, streptomycin, *Taq* DNA polymerase, dNTP, DNA kb ladder (GIBCO/BRL, Grand Island, NY); Superscript™ reamplification system for cDNA synthesis (GIBCO/BRL, Bethesda, MD); Micro-Fast Track mRNA isolation Kit (Invitrogen Corporation, San Diego, CA); Magic™ Miniprep DNA purification Kit, the fmol™ DNA Sequencing System (Promega, Madison, WI); restriction endonucleases (New England Biolabs, Bevcrly, MA); acrylamide (ACP, Montreal, QC); ammonium persulfate (APS) (Anachemia, Roses Point, NY); ethidium bromide (EtBr), N, N, N', N'-tetramethylethylenediamine (TEMED), (Fisher, Nepean, ON); agarose (Dalton Chemical, North York, ON); NuSeive low melting agarose (FMC, Rockland, ME).

2.4.2 Fibroblast culture

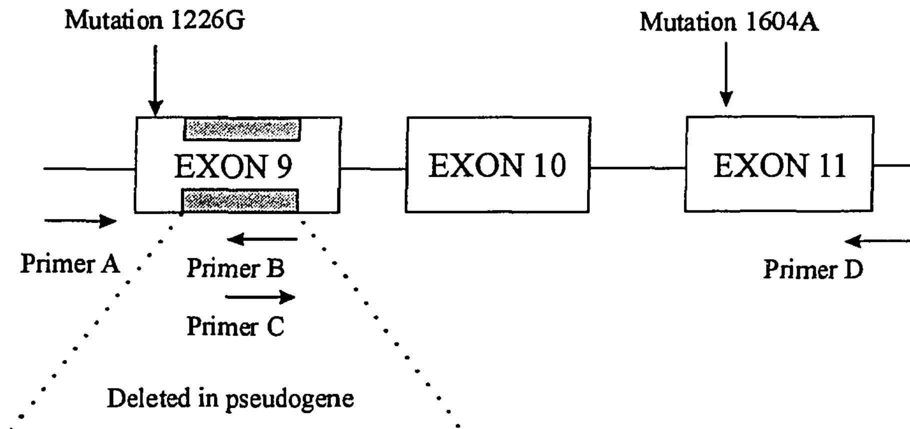
Fibroblasts of normal control and Gaucher patients were grown in RPMI-1640 medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100

μg/ml). The cultures were incubated at 37 ^{0}C in 5% CO_2. The confluent monolayers of fibroblasts were harvested and cell pellets were stored at -80 ^{0}C until use (Choy and Davison, 1978).

2.4.3 Genomic DNA isolation and PCR amplification

Genomic DNA was isolated from harvested fibroblasts using the TurboGen™ Genomic DNA Isolation Kit (Invitrogen Corporation, San Diego, CA). Genomic DNA was used as a template for the PCR amplification of exon 9 to exon 11 of the glucocerebrosidase gene. Four primers (primers A, B, C, and D; Figure 2.1) were designed to selectively amplify the partial glucocerebrosidase functional gene, whereas primers B and C are complementary the functional gene but not the pseudogene (Figure 2.1). Primers A and B flank the part of exon 9 where mutation 1226G is located. The posterior part of exon 9, exon 10 and exon 11 are flanked by primers C and D (Choy *et al.*, 1994a). The PCR reaction mixture was prepared as follows: 0.25 mM dNTP, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 1x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl). Approximately 0.5 μg of genomic DNA was add to the reaction mixture. The reaction mixture was incubated for 5 min at 94 °C for denaturation of the double-stranded DNA. *Taq* polymerase (2.5 units) was then added and a 30-cycle amplification was conducted in a DNA Thermal Cycler (Barnstead Thermolyne, Kirkland, WA). Each amplification cycle was 45 seconds at 94 °C for DNA denaturation, followed by 90 seconds at 58 °C for primer annealing, and 90 seconds at 72 ^{0}C for chain elongation.

To detect the presence of the common Gaucher disease-producing mutation 1226G, a genomic DNA fragment was amplified using a mismatch PCR method, and



Primer A: 5'GCCTTTGTCCTTACCCTCGA3'
Primer B: 5'GACAAAGTTACGCACCCAAT3'
Primer C: 5'ACTTTGTCGACAGTCCCATC3'
Primer D: 5'CTTTAATGCCCAGGCTGAGC3'

Figure 2.1 The primers used to selectively amplify exon 9 to 11 of the glucocerebrosidase structural gene. Primer A is sense to genomic nt 5801 to 5820; Primer B is antisense to nt 5926 to 5907; Primer C is sense to nt 5919 to 5938; Primer D is antisense to nt 6738 to 6719. The gray area in exon 9 indicates the sequences present in the functional gene, but not in the pseudogene (Choy *et al.*, 1994a).

analyzed using RFLP with *Xho*I digest (Beutler *et al*, 1990). The mismatch PCR utilized a 5' primer that mismatched at one nucleotide so as to create a *Xho*I restriction site for RFLP analysis of mutation 1226G (Figure 2.2).

2.4.4 mRNA isolation and cDNA synthesis

Poly-A mRNA was isolated from cultured fibroblasts using the Micro-FastTrack™ mRNA isolation kit. The first strand cDNA of the glucocerebrosidase gene was synthesized by the reverse transcription method using the cDNA Cycle™ kit with a gene specific primer (primer D, Figure 2.3). (Choy *et al.*, 1994a). Primers E and D (as shown in Figure 2.3) were used in the subsequent PCR amplification of the full length cDNA of glucocerebrosidase. Primers used in the PCR amplification of glucocerebrosidase cDNA were designed based on published cDNA sequences (Sorge, *et al.*, 1985; Tsuji *et al*, 1986).

2.4.5 Sequence analysis

Sequence analysis was performed using the fmol™ DNA sequencing system, based on the dideoxynucleotide chain termination method by Sanger *et al.* (1977). Primers used in the sequence analysis of glucocerebrosidase were shown on the Figure 2.3. γ-^{32}P dATP (NEN, Boston, MA) was used for end-labeling the sequencing primers. The reaction samples were run on a 6% acrylamide gel with 1xTBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 65W/2,000V. Autoradiography of the sequencing gel was carried out using BioMax™ film (Kodak, Rochester, NY) exposed overnight at room temperature.

2.4.6 RFLP analysis

PCR products were purified using the Magic™ DNA purification kit from Promega (Madison, WI). The restriction endonuclease digest of selected DNA fragments

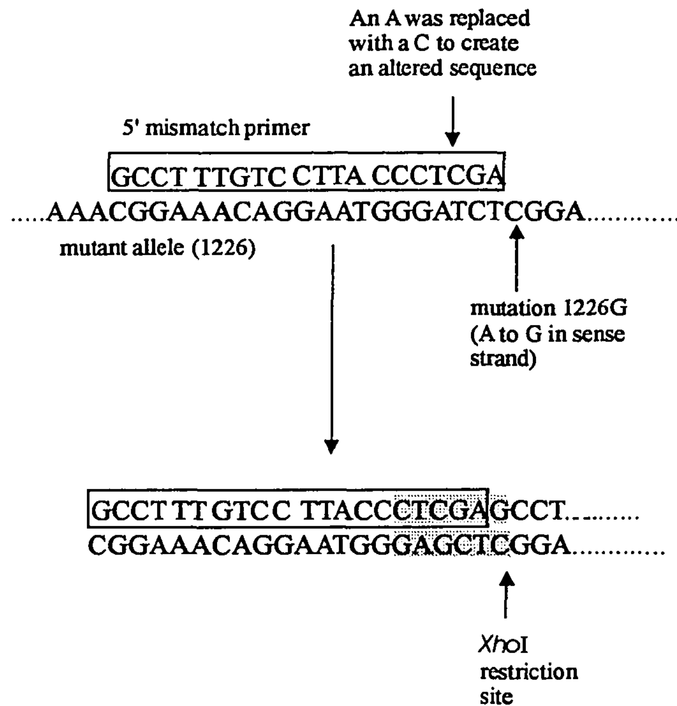
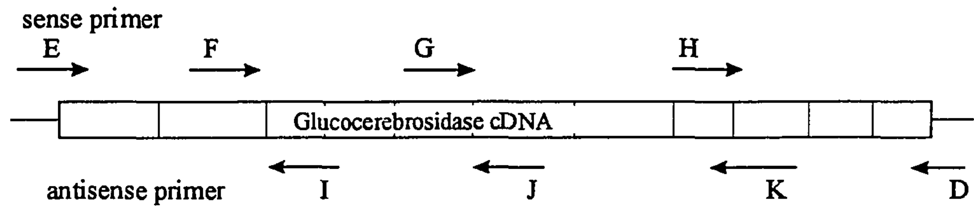


Figure 2.2 A mismatch PCR and *XhoI* RFLP to detect the presence of mutation 1226G using genomic DNA as a template. The mismatch primer is sense to genomic nt 5821- 5840. The downstream primer is 5'ACAA-AGTTACGCACCCAAT3', that is antisense to genomic nt 5926 - 5906.



Primer D: 5'-CTTTAATGCCCAGGCTGAGC-3'
Primer E: 5'-TATCAGATCTTCATCTAATGACCCTGA-3'
Primer F: 5'-CTGCTGCTCTCAACATTCTT-3'
Primer G: 5'-TACAGTTCTGGGCAGTGACA-3'
Primer H: 5'-ATCATCACGAACCTCCTGTA-3'
Primer I: 5'-ATAGGTGTAGGTGCGGATGGA-3'
Primer J: 5'-GAAGCGGTATCCACTCAACA-3'
Primer K: 5'-GACAAAGTTACGCACCCAAT-3'

Figure 2.3 Primers for sequence analysis of full length glucocerebrosidase cDNA.

was performed according to the manufacture's suggestion. To verify the sizes of DNA fragments, the digested DNA mixtures, with a DNA kb ladder, were subjected to electrophoresis at 25mA on a 10% acrylamide gel with 1x TBE buffer. The mini-gel apparatus from Owl Scientific (Cambridge, MA) was used for the acrylamide gel electrophoresis. After the electrophoresis, the acrylamide gel was incubated with EtBr (5 μg/ml in ddH_2O) for 10 min, followed by destaining with ddH_2O for 10 min. The gel was then photographed on the top of a UV illuminator (Fisher Scientific, Toronto, ON).

2.5 Results

Patient BD. Genomic DNA of glucocerebrosidase exon 10 amplified by the PCR method from patient BD was subjected to sequence analysis. The presence of a T to C missense mutation in the heterozygous form at cDNA nt 1448C was noted in patient BD. Using *NciI* RFLP analysis, Choy *et al.* (1991) previously reported that this patient was heterozygous for this mutation. Data from our present sequence analysis confirm this finding.

Since the remaining nucleotide sequence of exon 10 from patient BD was identical to that of the control, we continued to search for the unknown mutation(s) in the other Gaucher allele by sequencing glucocerebrosidase cDNA from patient BD. A heterozygous C to T transition in cDNA nt 649 was noted (Figure 2.4). The mutation 649T results in Pro to Ser substitution in amino acid 178 of glucocerebrosidase. In addition, two heterozygous silent mutations - an A to T transversion at cDNA nt 114 and a C to T transition at cDNA nt 189, were also detected (data not shown). In order to rule out the possibility of multiple point mutations that may be present among some Gaucher patients (Latham *et al.*, 1990; Zimran *et al*, 1990), we performed sequence analysis of the

Mutant Normal
A C G T A C G T

C
C
Pro/Ser C/T

C
C
C Pro

Figure 2.4 Sequence analysis and identification of mutation 649T from glucocerebrosidase cDNA of patient BD. The nucleotide sequences of the sense strand of the control are shown in the four right lanes and that of patient BD are shown in the four left lanes. A heterozygous C to T mutation (CCC/T instead of CCC) was noted in cDNA nt 649 of the patient (Choy and Wei, 1995).

full-length cDNA of the glucocerebrosidase gene. The nucleotide sequences in the rest of glucocerebrosidase cDNA were normal (Choy *et al.*, 1995).

A computer search for endonuclease cleavage sites using the DNASIS V 5.0 Program (Pharmacia LKB, Sweden) showed that the mutation at cDNA nt 649 has abolished a *BsajI* cleavage site. Subsequently, the presence of mutation 649T in patient BD was confirmed by RFLP analysis using *BsajI*. In the control, there are three naturally occurring *BsajI* cleavage sites at cDNA nt 482, 650, and 740 within the cDNA fragment. After a *BsajI* digestion, DNA bands of 168, 116, 104, and 90 bp were found (Figure 2.5, lane 3 from left). In patient BD, mutation 649T altered the specific enzyme recognition site required for cleavage by *BsajI*, resulting in the presence of an undigested band of 258 bp (lane 2, Figure 2.5). Similar results of *BsajI* RFLP analysis were obtained using PCR amplified genomic DNA (data not shown).

Patient BL. Exon 10 of the glucocerebrosidase gene amplified by the mismatch PCR method from patient BL was subjected to *XhoI* RFLP analysis. As shown in Figure 2.6, in the normal control, there is no cleavage site for the restriction enzyme *XhoI*. Subsequently, the presence of one undigested band of 105 bp was noted (Figure 2.6, lane 2 from left). In the positive control, which is homozygous for this mutation, digested bands of 86 and 19 bp were found (Figure 2.6, lane 5). In patient BL, the presence of both the 86 and 105 bp bands was noted (Figure 2.6, lane 4). This finding demonstrated that the patient is heterozygous for mutation 1226G, the most common mutation in Gaucher disease (Beutler *et al.*, 1992).

The mutation 1226G was also confirmed by sequence analysis. The presence of an A to G transition in the heterozygous form was noted at cDNA nt 1226. In view of

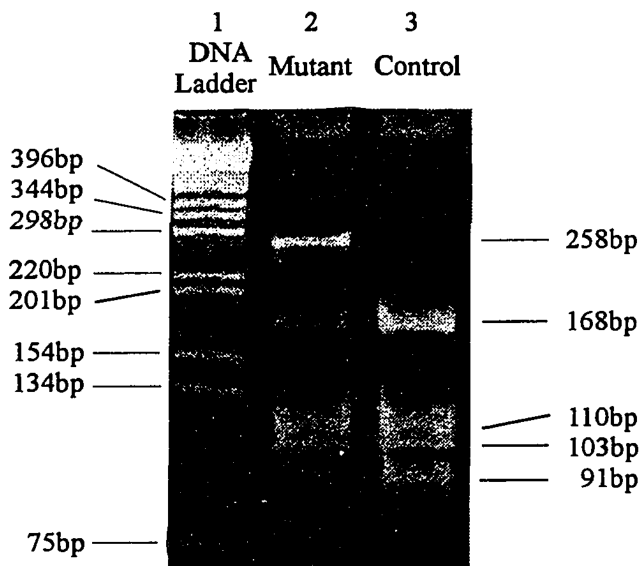


Figure 2.5 *BsajI* RFLP analysis of mutation 649T. cDNA sample of patient BD was amplified by the PCR method and digested by *BsajI* endonuclease. The upstream primer is 5'-CTGCTGCTCTCAACATCCTT-3', which is sense to cDNA nt 380 to 399. The downstream primer is 5'-GAAGGGGTATCCACTCAACA-3', which is antisense to cDNA nt 855 to 836. From left: Lane 1, 1kb DNA ladder (GIBCOL/BRL). Lane 2, the mutation at cDNA nt position 649 of patient BD has abolished a cleavage site for *BsajI* endonuclease, resulting in the presence of an undigested bands of 258 bp. Digested bands of 90 and 168 bp were also found in the patient's sample, confirming that mutation 649T is present in the heterozygous form. Lane 3, a normal control (Choy and Wei, 1995).

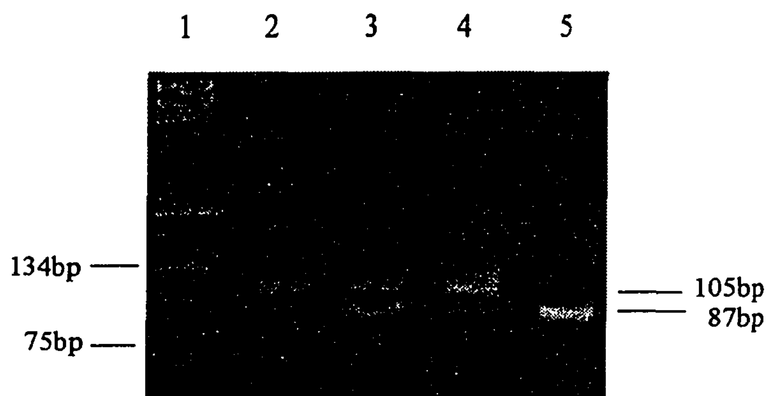


Figure 2.6 *Xho*I endonuclease digestion and PAGE of genomic DNA of glucocerebrosidase exon 9 amplified by the mismatch PCR method. From left to right: Lane 1, DNA ladder (GIBCO/BRL). Lane 2, normal control, negative for the 1226G mutation and *Xho*I cleavage site. Lane 3 and 4, heterozygous 1226G mutant alleles. Lane 5, homozygous 1226G mutant alleles (Choy *et al.*, 1994a).

thereport that mutation 1226G is present only in type 1 Gaucher disease (Zimran *et al.*, 1989), this finding further confirms the diagnosis of patient BL as having the non-neuronopathic type 1 form.

The mutation in the other Gaucher allele was also identified during sequence analysis. As shown in Figure 2.7, there was a T to G transversion in the heterozygous form at cDNA nt 1366, resulting in Phe to Val substitution at amino acid 417 of glucocerebrosidase. A computer search for endonuclease cleavage sites showed that the mutation has created a new *Nco*I site. The presence of mutation 1366 was subsequently confirmed by *Nco*I RFLP analysis. As shown in Figure 2.8, two bands of 226 and 592 bp were found in the control because of a naturally occurring *Nco*I cleavage site at genomic nt 6146. In patient BL, mutation 1366G creates a second *Nco*I site. It results in the cleavage of the band of 226 bp to two bands of 61 and 165 bp. Since both the 226 and 165 bp bands are present (lane 2, Figure 2.8), it demonstrates that mutation 1366G is present in the heterozygous form (Choy *et al.*, 1994b).

We detected the presence of a heterozygous silent mutation at cDNA nt 84 (G to T transversion) at the degenerate position of the codon for glycine, and a heterozygous base substitution at cDNA nucleotide position 1601 (A to G transition) that results in His to Arg substitution at amino acid 495 of glucocerebrosidase. The later finding is of interest because it was previously reported that this may be a cloning artifact (Beutler *et al.*, 1988) or a neutral mutation among normal individuals (Wigderson *et al.*, 1989). Choy *et al.*, (1997) screened for this particular codon in 256 subjects without Gaucher disease and 15 Gaucher disease patients. They found the 1601G genotype was present in the homozygous form in all of the asymptomatic subjects and 14 Gaucher patients. The

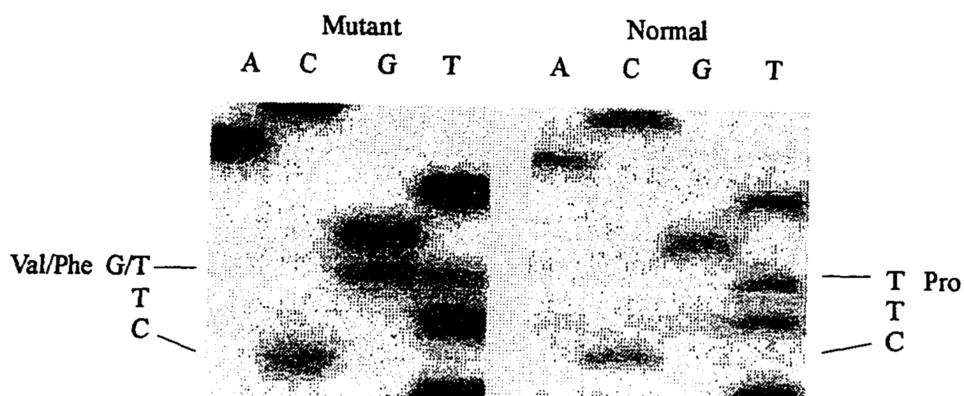


Figure 2.7 Sequence analysis of PCR-amplified glucocerebrosidase cDNA from a control and Gaucher patient BL. A T to G missense mutation in the heterozygous form at cDNA nucleotide position 1366 of patient BL was noted (Choy *et al.*, 1994b).

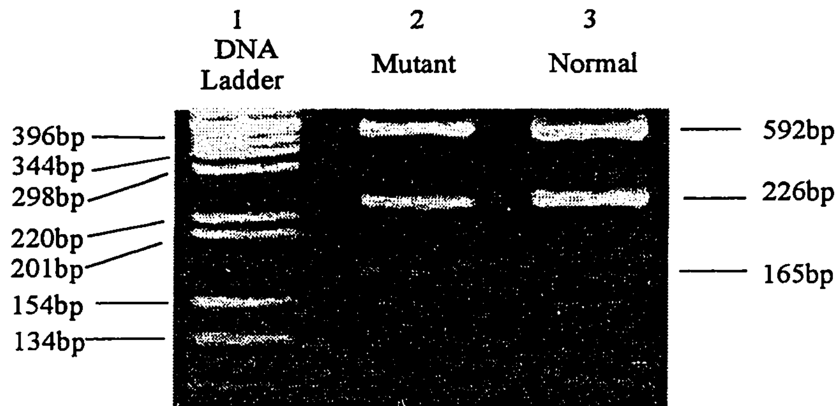


Figure 2.8 *Nco*I RFLP analysis of mutation 1366G using genomic DNA as a template. The primers are shown on Table 2.2. From left to right: Lane 1, 1kb DNA ladder (GIBCOL/BRL). lane 2, mutant allele of patient BL. Lane 3, normal control. The heterozygous mutation 1366G has created a new cleavage site for *Nco*I endonuclease and subsequently the presence of two additional bands at 165bp and 61bp. The band of 165bp was shown on the figure (Choy *et al.*, 1994b).

1604A genotype was only detected in one Gaucher patient (BL) in a heterozygous form (1604G/A). This finding indicated that 1601G genotype which encodes Arg at amino acid position 495 of the glucocerebrosidase polypeptide is not a cloning error. Instead, it constitutes the normal as well as predominant genotype in the population tested (Choy *et al.*, 1997). Sequence analysis of the rest of the glucocerebrosidase cDNA showed that the DNA sequences are identical to that of the published sequences from normal individuals (Horowitz *et al.*, 1989).

Patient ES. The results of the mismatch PCR and the *Xho*I restriction analysis revealed the presence of a heterozygous form of the mutation 1226G in the glucocerebrosidase gene of ES (data not shown). In order to confirm the above observation, genomic DNA of glucocerebrosidase exon 9 amplified by the PCR method from the patient was subjected to sequence analysis. The presence of an A to G missense mutation at cDNA nt 1226 was noted.

A mutation in the other Gaucher allele of patient ES was identified by sequence analysis of the cDNA of the glucocerebrosidase gene. As shown in Figure 2.9, there was a G to A transition in the heterozygous form at cDNA nt 1604, resulting in Arg to His substitution at amino acid 496 of glucocerebrosidase. The nucleotide sequence in the rest of the full-length cDNA was identical to that of the published sequence from normal individuals (Tsuji *et al.*, 1986).

The mutation 1604A creates a new *Hph*I site. The presence of the mutation 1604A in the patient was confirmed by digesting PCR amplified cDNA samples of the patient and control with *Hph*I endonuclease and analysis in an acrylamide gel. As shown in Figure 2.10 (lane 3 from left), two bands of 256 and 99 bp were found to be in the digested DNA of

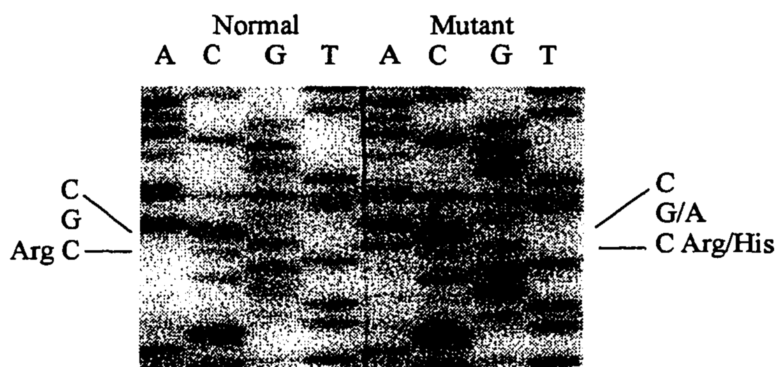


Figure 2.9 Sequence analysis of PCR-amplified glucocerebrosidase cDNA. The four lanes from the left are the A, C, G, and T lanes of a control. The four lanes from the right are those of patient ES. As shown, there is a G to A missense mutation in the heterozygous form at cDNA nucleotide position 1604 in patient ES (Choy *et al.*, 1994a).

normal control. The heterozygous mutation 1604A resulted in two additional bands (65 and 34 bp). The band of 65 bp was distinguishable on the Figure 2.10, lane 2. Similar results from RFLP analysis were noted when genomic DNA instead of cDNA was used as the template for PCR amplification (Choy *et al.,* 1994a).

Patient JB. The mutation 1226G was not detected from genomic DNA of patient JB by using the mismatch PCR and the *XhoI* endonuclease digestion. The full length of glucocerebrosidase cDNA of JB was subjected to sequence analysis. As shown in Figure 2.11, there was a C to T transition in the homozygous form at cDNA nt 481, resulting in Pro to Ser substitution at amino acid 122 of glucocerebrosidase. The nucleotide sequence in the rest of the full-length cDNA was identical to that of the published sequence from normal individuals (Tsuji *et al.,* 1986).

It was noted that the mutation abolished a *Kpn*I digestion site. The presence of mutation 481T in the patient was confirmed by digesting PCR-amplified cDNA samples of the patient and control with *Kpn*I endonuclease and following by the PAGE analysis. As shown in Figure 2.12, two bands of 1058 and 748 bp were found in the control (lane 3 from left). Whereas in the digested DNA of the patient (Figure 2.12, lane2), there was only one band at 1806 bp. It showed that mutation 481T was present in the homozygous form. Similar results from RFLP analysis were noted when cDNA instead of genomic DNA was used as the template for PCR amplification.

Patient CR. The results of direct cDNA sequence analysis showed the presence of the heterozygous mutation 1226G and 1448C in the glucocerebrosidase gene of patient CR. The nucleotide sequence in the rest of the full-length cDNA was identical to that of the published sequence from normal individuals.

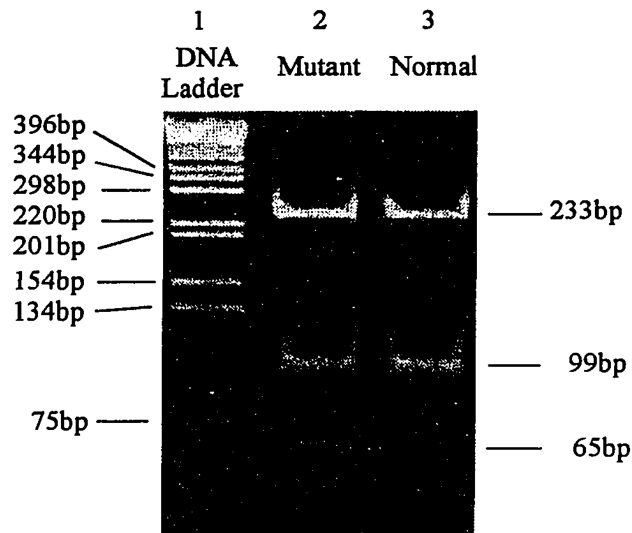


Figure 2.10 *HphI* RFLP analysis of mutation 1604A. The cDNA of patient ES was amplified by the PCR method as described in the text. The primers that used for the PCR amplification are primer C and D as described in Figure 2.1. From right to left: Lane 1, 1kb DNA ladder (BIBCOL/BRL). Lane 2, sample of patient. Lane 3, normal control. The heterozygous mutation at cDNA nucleotide position 1604 of the patient ES has created a new site for *HphI* endonuclease, resulting in two additional bands of 65 and 34 bp from the band of 99bp. The band of 34bp was too faint to be shown in the picture (Choy *et al.*, 1994a).

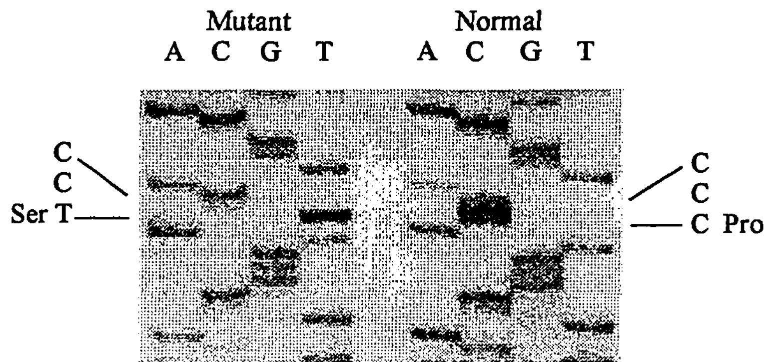


Figure 2.11 Sequence analysis of PCR-amplified glucocerebrosidase cDNA from a control and Gaucher patient JB. A C to T missense mutation in the homozygous form at cDNA nucleotide position 481 of patient JB was noted.

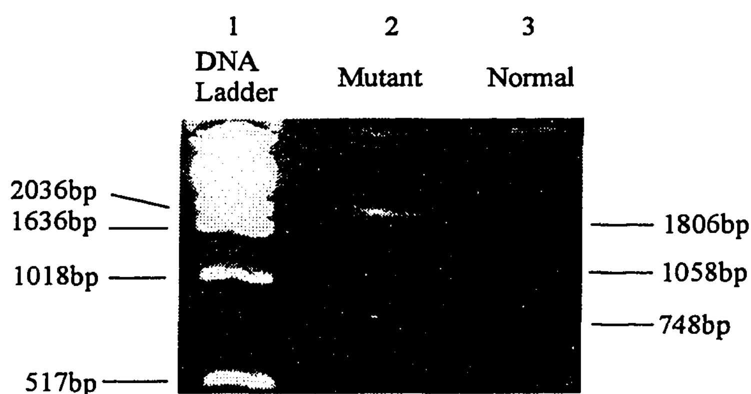


Figure 2.12 *Kpn*I RFLP analysis of mutation 481T. The genomic DNA of patient JB was amplified by the PCR method as described in the text. The primers that used for the PCR amplification are primer M and N as described is Table 2.2. From right to left: Lane 1, 1kb DNA ladder (BIBCOL/BRL). Lane 2, sample of patient. Lane 3, normal control. The homozygous mutations at cDNA nt 481 of the patient JB have abolished a digestion site for *Kpn*I endonuclease, resulting in a band of 1806bp.

As described previously, the presence of mutation 1226G can also be detected by digesting PCR-amplified cDNA (or genomic DNA) samples of the patient and control with *Xho*I endonuclease and analysis in PAGE. The mutation 1448C also creates an endonuclease *Nci*I recognition site. Therefore, a similar approach of the RFLP analysis can be used to detect the presence of the mutation 1448C, with endonuclease *Nci*I.

Patient VD. The result of the mismatch PCR and the RFLP analysis using the endonuclease *Xho*I showed the presence of a homozygous form of the mutation 1226G in the glucocerebrosidase gene of patient VD. Therefore, no further direct sequence analysis was performed for this patient.

2.6 Discussion

The results of this study are summarized in Table 2.1. We performed direct sequence analysis on PCR-amplified glucocerebrosidase cDNA synthesized by reverse transcription. This procedure by-passed the gene cloning step and eliminated the possibility of errors arising from gene cloning. The possibility of errors resulting from *Taq* polymerase is also unlikely since consistent reproducible findings were noted using cDNA samples that were PCR-amplified in separate experiments. Mutations identified by cDNA sequence analysis were also confirmed by sequence analysis of the specific fragments of genomic DNA in each individual and by RFLP analysis of genomic DNA or cDNA samples.

All of the mutations identified in this study can be confirmed by RFLP analysis with various endonucleases. The specific primers were designed to amplify the mutation-containing fragments of genomic DNA using PCR amplification (Table 2.2). The combination of PCR amplification and RFLP analysis has enabled us to screen for

Patients	Clinical forms	Genotype [a]	Mutations	nt position in genomic DNA	Amino acid substitutions
BD	2	649T	CCC → TCC	3443	Pro178Ser
		1448C	CTG → CCG	6533	Leu444Pro
BL	1	1226G	AAC → AGC	5841	Asn370Ser
		1366G	TTC → GTC	5981	Phe417Val
JB	1	481T/481T	CCC → TCC	3065	Pro122Ser
ES	1	1226G	AAC → AGC	5841	Asn370Ser
		1604A	CGC → CAC	6683	Arg496His
VD	1	1226G/1226G	AAC → AGC	5841	Asn370Ser
CR	1	1226G	AAC → AGC	5841	Asn370Ser
		1448C	CTG → CCG	6533	Leu444Pro

[a] The number is the nucleotide number of glucocerebrosidase cDNA.

Table 2.1 Summary of glucocerebrosidase gene mutations identified from six Gaucher patients.

Mutations [a]	Endonuclease digestion sites altered [b]	Primers [c] for PCR amplification of genomic DNA fragments	Positive control
649T	-*Bsaj*I	L & N	BD
1366G	+*Nco*I	C & D	BL
481T	-*Kpn*I	M & N	JB
1604A	+*Hph*I	C & D	ES

[a] The number is the nucleotide number of glucocerebrosidase cDNA.

[b] + The mutation results in a new endonuclease digestion site.
- The mutation abolishes an endonuclease digestion site.

[c] Primer C & D are shown on Figure 2.1.
Primer L: 5'TCCATCCGCACCTACACCTAT3'
Primer M: 5'CTGCTGCTCCAACATCCTT3'
Primer N: 5'TGGGTGACAGAGAGAGACT3'

Table 2.2 Summary of the RFLP analysis of mutation 649T, 1366G, 481T and 1604A. Primer L is sense to genomic nt 3090 to 3111; Primer M is sense to genomic nt 2007 - 2026; Primer N is antisense to genomic nt 3813 - 3794, where the sequence is absent in the pseudogene.

the presence of those mutations with precision among Gaucher patients.

Mutation 1226G is the most common mutation in Gaucher disease and it accounts for about 77% of the total mutations in Jewish patients (Beutler *et al.*, 1992b). It is postulated that homozygous mutation 1226G will result in mild clinical presentation, delayed onset of symptoms and good prognosis (Beulter *et al.*, 1992b; Zimran *et al.*, 1989; Sibille *et al*, 1993). Identification of this mutation is of prognostic value as it is present only in type 1 Gaucher disease (Choy *et al.*, 1991; Zimran *et al.*, 1989). However, when it is present in the heterozygous form with either mutation 1448C, exon 2 insertion mutation or intron 2 splicing mutation, the clinical severity scores assigned to the patients are considerably higher (Beulter *et al.*, 1992b). The observations that there were no neurological involvement in our patients ES, CR and VD were consistent with the postulate that the mutation 1226G usually results in type 1 Gaucher disease. Based on this study and other reports, mutation 1226G appears to be a common mutation among non-Jewish European populations as well (Amaral *et al.*, 1993. Choy *et al.*, 1994a, 1994b; Cormand *et al.*, 1995; Coutre *et al.*, 1997). However, it is not a common mutation among the Asian population (Eto *et al.*, 1993; Kim *et al.*, 1996; Choy *et al.*, 1997).

For Gaucher patient BD, we have identified a new missense (T to A) mutation in cDNA nt 649 that results in Pro to Ser substitution at amino acid 178 of glucocerebrosidase. The presence of heterozygous mutation 1448C in this infantile patient was also confirmed by sequence analysis. This 649T/1448C genotype appears to result in a poor prognosis, as the patient had died with type 2 Gaucher disease at the age of 18 months. By comparison, Gaucher patients with the 1226G/1448C genotype do not have neurological involvement, and may have very mild clinical onset (Zimran *et al.*, 1989;

Choy *et al.*, 1991; Sibille *et al.*, 1993). This observation suggests that mutation 649T may result in more severe clinical involvement than mutation 1226G, which usually results in good prognosis (Zimran *et al.*, 1993). Mutation 1448C is one of the most common mutations in Gaucher disease among non-Jewish patients (Walley *et al.*, 1993; Horowitz *et al.*, 1993; Eto *et al.*, 1993; Kim *et al.*, 1996). It accounts for 37% of the 128 mutant alleles in a survey among non-Jewish Gaucher patients (Beutler *et al.*, 1993). In a parallel study on the relationship between age and severity of symptoms of 120 unrelated patients with several common Gaucher disease mutations, patients with the 1448C/1448C genotype had the most severe symptoms (Beutler *et al.*, 1993).

Mutation 1604A is a G to A transition in the nondegenerate position of the codon (CGC to CAC) that results in Arg to His substitution at amino acid 496 of glucocerebrosidase. Arg is a basic amino acid with a guanidine group of pKa value 12.5, whereas His is a relatively neutral amino acid with an imidazole group of pKa value 6.0, the substitution of His for Arg at position 496 near the enzyme active site (Diniur *et al.*, 1989) would be expected to have profound effects on the biophysical and catalytic properties of glucocerebrosidase. However, the fact that patient ES has no neurological involvement at age 46 suggests the mutation 1604A, in conjunction with mutation 1226G in the other Gaucher allele, is also consistent with type 1 Gaucher disease. It was also reported (Beutler *et al.*, 1993a) that the mutation at nucleotide 1604A was present together with the frameshift 84GG mutation in three unrelated families and mutation 1226G in another family. No neuronopathic disease was observed and the course of the patients was relatively mild (Beutler *et al.*, 1993a).

For patient BL, the splenectomy performed more than fourteen years ago on the patient apparently reversed the hematological complications and there has been no history of hepatic or neurological involvement. The clinical course of Gaucher disease in this patient with the 1226G/1366G genotype so far has been a mild one. Therefore, it can be classified as type 1 Gaucher disease. Since this is the first report of the identification of mutation 1366G in a Gaucher patient, it is hard to predict the outcome of the other genotypes, such as 1366G/1448C, 1366G/1366G or 1366G/84GG. Further study needs to be done for this novel mutation (Choy *et al.*, 1994b).

It is interesting to notice that the mutation 481T was only identified from Gaucher patients with native Indian origin. In a separate report, this mutation found in the homozygous form produced severe visceral disease (Beutler *et al.*, 1993a). In these two cases (our patient BL and Beutler's report), the patients were diagnosed at young age without neurological involvement. It is difficult to draw conclusions about the severity of this mutation at present. Since this mutation appears to be unique among the aboriginal population, it should be one of the main considerations for mutation screening or for genetic counseling. A survey and estimation of the frequency of this mutation among this particular population will provide further information for this unique mutation.

Chapter 3 Characterization of Glucocerebrosidase Using The Baculovirus Expression System

3.1 Abstract

Two novel mutations (649T and 1366G) and two rare mutations (481T and 1604A) in the glucocerebrosidase gene of Gaucher patients were described in the previous chapter. To demonstrate that those mutations are deleterious and not neutral mutations, we cloned the full-length normal and mutant glucocerebrosidase cDNA into plasmid vector pAcUW1 at a *Bgl*II site downstream from the *p10* promoter of the baculovirus *Autographa californica* nucleopolyhedrovirus (*AcNPV*). Recombinant plasmids were used to co-transfect with the DNA of baculovirus *AcUW1.lacZ* into *Spodoptera frugiperda* cells (*Sf9*) using the calcium phosphate-mediated transfection method. Recombinant baculovirus was purified by the plaque purification method. Glucocerebrosidase activities from crude extracts of transfected *Sf9* cells were assayed using the fluorogenic substrate 4MUGP. The levels of residual glucocerebrosidase activity determined in transfected cells with the Gaucher 649T, 1366G, 481T, and 1604A alleles are 2.8%, 2.9%, 17.3% and 6.9% of that expressed by the normal allele (normal = 352.0 nmol/hr/mg protein). By comparison, the enzyme-specific activity expressed in transfected cells by 2 known Gaucher alleles, 1226G and 1448C, that are prevalent in type 1 and type 2 Gaucher disease were 23.4% and 3.3% of normal. No endogenous glucocerebrosidase activity was detected in culture cells transfected by wild type baculovirus. The results of this study demonstrated that the two novel mutations (1604A and 1366G) and the two rare mutations (481T and 1604A) are deleterious resulting in profoundly deficient

glucocerebrosidase activity and subsequent Gaucher disease. The clinical phenotypes may be determined by the combination of different mutant alleles.

3.2 Introduction

In the previous Chapter, we described the sequence analysis of mutant glucocerebrosidase alleles from six unrelated Gaucher patients. Two novel mutations (649T and 1366G) and two rare mutations (481T and 1604A) were identified. To demonstrate unequivocally that mutations 649T, 1366G, 481T and 1604A are deleterious and result in deficient glucocerebrosidase activity and subsequent Gaucher disease, it is essential to express these mutant alleles in a suitable system and determine the level of glucocerebrosidase activity expressed.

The baculovirus expression system (Figure 3.1) is an eukaryotic expression system that is capable of performing eukaryotic post-translational modifications similar to those in mammalians. Since the first baculovirus vector was developed in 1983 (Smith *et al.*), this expression system has been widely used for the heterologous expressions of various proteins. baculovirus *AcUW1.lacZ* is a modified *AcNPV* (*Autographa californica* nucleopolyhedrovirus) baculovirus that contains a *p10* promoter, partial *p10* gene sequences, polyhedrin gene, and *lacZ* gene (Gruenwald and Heitz, 1993). The *p10* promoter is a strong promoter (O'Reilly *et al.*, 1992) and foreign genes inserted downstream of this promoter can be expressed at high levels. The *p10* gene is non-essential for viral infection and replication under tissue culture conditions (O'Reilly *et al.*, 1992). Therefore, at the *p10* gene locus a *lacZ* gene was placed as a colour selectable marker (Gruenwald and Heitz, 1993). *AcUW1.lacZ* DNA was linearized to increase the recombination frequency when co-transfected with a *p10* locus-based

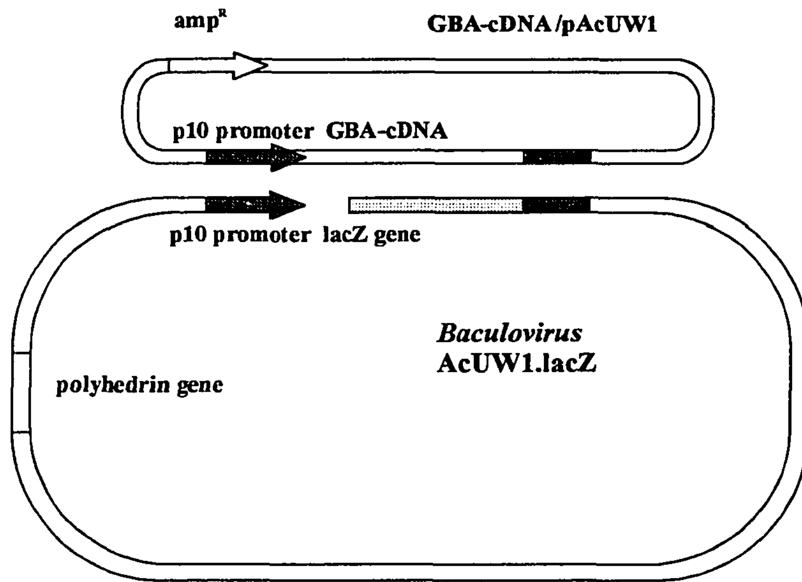


Figure 3.1 Strategy for the introducing the glucocerebrosidase (GBA) cDNA from plasmid pAcUW1 into the *Baculovirus* genome by an allelic exchange precess after calcium phosphate-mediated cotransfection of cultured *Sf9* cells. The deep gray areas are the homologous regions of DNA in the vector and *Baculovirus* genome respectively (Choy *et al.*, 1996).

baculovirus transfer plasmid. The full-length glucocerebrosidase cDNA was cloned into the baculovirus transfer plasmid pAcUW1 and cotransfected with baculovirus *AcUW1.lacZ* DNA into *Spodoptera frugiperda* cells (*Sf9*). When a double cross-over event occurred between homologous *p10* gene DNA, the glucocerebrosidase gene recombined into the baculovirus genome. The propagation of recombinant baculovirus in cultured *Sf9* cells will result the production of a recombinant protein.

Martin *et al.* (1988) as well as Grabowski *et al.* (1989) expressed recombinant glucocerebrosidase using the baculovirus expression system. They demonstrated that the baculovirus expression system is a suitable system for the expression of functional glucocerebrosidase and the recombinant enzyme expressed in this system has kinetic properties similar to that of the native enzyme. Furthermore, there is no endogenous glucocerebrosidase activity in cultured *Sf9* cells (Grace *et al.*, 1994) and very low levels of glucocerebrosidase activity expressed by a Gaucher allele can be detected. In this study, we cloned and expressed those mutant alleles and two known Gaucher alleles (1226G and 1448C), as well as a normal allele, in the baculovirus (*AcNPV*) expression system in transfected *Sf9* cells. Glucocerebrosidase activities from crude extracts of transfected *Sf9* cells were assayed using the fluorogenic substrate 4MUGP. The levels of enzyme activity determined were compared with that expressed by a normal allele and other known Gaucher alleles in the same expression system (Choy *et al.*, 1996).

3.3 Materials and methods

3.3.1 Fibroblast cell lines, *E. coli* strain, and *Spodoptera frugiperda* cells

Fibroblast cell lines established from skin biopsies of a normal control and

Gaucher patients BD, JB, BL, and ES were used in this study. The patients' clinical information, as well as methods for culturing and harvesting fibroblasts, were described in the previous chapter.

Escherichia coli strain Top 10F' from Invitrogen (San Diego, CA) and *Spodoptera frugiperda Sf9* cells from Dr. David Levin, Dept. of Biology, University of Victoria, were used in this study.

3.3.2 Chemicals and reagents

Micro-Fast Track mRNA isolation kit (Invitrogen Corporation, San Diego, CA); Magic™ miniprep DNA purification kit (Promega, Madison, WI); Superscript™ reamplification system for cDNA synthesis, fetal bovine serum, X-Gal (5-bromo-4-chloro-3indolyl β-D-galactoside), Fungizone, and Grace cell culture powder media (GIBCO/BRL, Bethesda, MD); 4-methylumbelliferyl-β-D-glucopyranoside (4MUGP), bovine serum albumin, Triton X-100, sodium cholate (Sigma, St. Louis, MO); *Taq* DNA polymerase (GIBCO/BRL, Grand Island, NY); sodium taurocholate (Cal Biochem, La Jolla, CA); trisodium citrate, citric acid (Caledon Laboratories, Georgetown. ON); restriction endonucleases (New England Biolabs, Beverly, MA); ammonium sulfate (BDH, Toronto, ON); ethylene glycol, sodium bicarbonate (Anachemia, Roses Point, NY); NuSeive low melting agarose (FMC, Rockland, ME).

The transfer vector pAcUW1, wild type baculovirus *AcNPV*, and linearized *AcUW1.lacZ* DNA were generously provided by Dr. David Levin.

3.3.3 Media and buffers

***Sf9* cell culture medium**: 0.33% Bacto Tryptone, 0.33% yeastolate, 0.035% $NaHCO_3$, 1 x Grace cell culture powder media, and adjusted pH to 6.2 with NaOH. Filter

sterilized before adding 10% heat inactivated fetal bovine serum and 1% Fungizone. **Liquid LB medium**: 1% Bacto Tryptone, 0.5% Bacto yeast extract and 1% NaCl, autoclaved. **Cracking buffer**: 50 mM Tris-HCl pH 7.5, 2 mM EDTA, 1% SDS (w/v), 13.7% sucrose, 0.01% bromophenol blue and 0.01% xylene cyanol. Filtered for sterilization. **CTAB (hexadecytrimethyl-ammonium bromide) virus DNA extraction Buffer**: 100 mM Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% β-mercaptoethanol.

3.3.4 mRNA isolation and cDNA synthesis

The mRNA from four Gaucher patients and one normal individual was isolated using Micro-Fast Track mRNA isolation kit (Invitrogen Corporation) and cDNA was synthesized using Superscript™ reamplification system for cDNA synthesis (GIBCOL/BRL). Mutant cDNA were confirmed by sequence analysis and RFLP analysis as described in chapter 2. Six mutant glucocerebrosidase cDNA (649T, 1366G, 481T, 1604A, 1226G, and 1448C) were used in this study.

3.3.5 Construction and selection of recombinant vector

To incorporate endonuclease digestion sites into the appropriate glucocerebrosidase cDNA, the full length glucocerebrosidase cDNA was PCR-amplified by using the sense primer 5'-TATC**AGA**<u>**T**CTTCATCTAATGACCCTGA</u>-3' (primer I) and the antisense primer 5'-AGTAG**AGAT**<u>**CT**TTAATGCCCAGGCGAGC</u> -3' (primer II). For primer I, the nucleotides underlined are sense to genomic nucleotide positions 560 - 579 and cDNA nucleotide positions minus 23 - minus 4 (Tsuji *et al.*, 1986). Those of the primer II are antisense to genomic nucleotide positions 6738 - 6719 and cDNA nucleotide positions 1658 - 1639 (Tsuji *et al.*, 1986). AGATCT (bold) are the *Bgl*II recognition sequences for both primers. After the PCR amplification, the cDNA was

electrophoresed on a 1.5% NuSeive low melting agarose gel. The gel portion containing cDNA was cut out and purified using the Magic™ DNA purification Kit. The purified cDNA fragment was then digested by *Bgl*II endonuclease, annealed, and ligated to the *Bgl*II site of pAcUWl downstream of the *AcNPV p10* promoter to create the recombinant transfer vector cDNA/pAcUW1. The recombinant vector was transferred into the *E. coli* strain Top 10F' using the calcium chloride transformation method (Graham and VanDereb, 1973). Clones containing the glucocerebrosidase cDNA insert were selected using the cracking assay method. In brief, ampicillin resistance clones were transferred to a fresh LB plate 12 hr prior the cracking assay. Bacterial cells were transferred to a microcentrifuge tube containing 50 μl of cracking buffer. After incubation at room temperature for 20 min, the samples were loaded in a 0.7% agarose gel for electrophoresis. The banding patterns of recombinant clones were compared to the control clone that contained a non-recombinant vector only. Positive clones were selected for further amplification. To select a recombinant vector that contained the glucocerebrosidase cDNA insert with correct orientation (Figure 3.2), the recombinant clones of *E. coli* Top 10F' were cultured at 37 ^{0}C in LB liquid medium overnight. The plasmid DNA was extracted and purified using the Magic™ miniprep DNA purification kit. The plasmid DNA was then digested with restriction endonucleoase *Xho*I and *BamH*1 at 37 ^{0}C overnight. The digested plasmid DNA was run on a 0.7% agarose gel to verify the presence and orientation of glucocerebrosidase cDNA insert.

3.3.6 Generation of recombinant baculovirus

The purified recombinant vector cDNA/pAcUW1 was co-transfected with the linearized *AcUW1.lacZ* DNA into host cells using the calcium phosphate-mediated

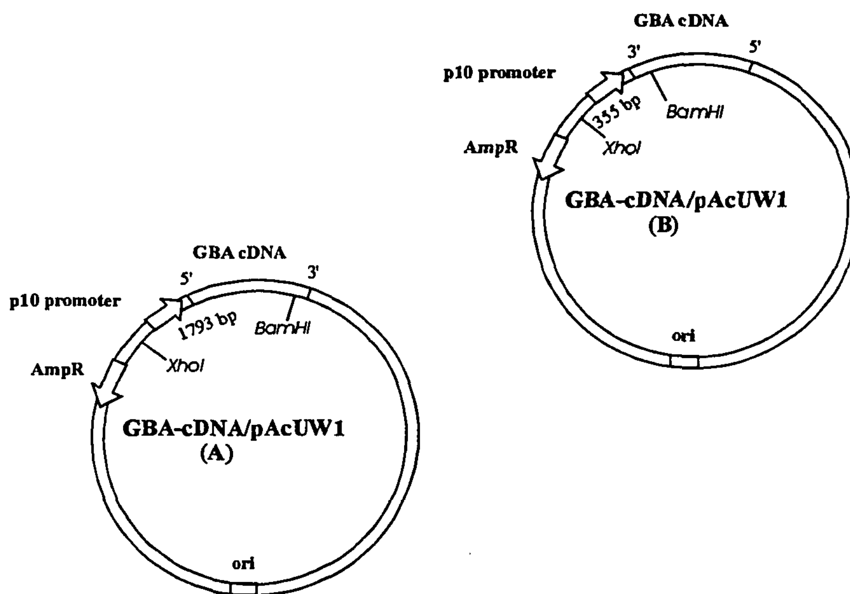


Figure 3.2 Strategy for ligating and cloning the glucocerebrosidase (GBA) cDNA insert in the *Baculovirus* plasmid vector pAcUW1. If the cDNA insert is inserted in the 5' to 3' orientation, the *Xho*I and *BamH*I digests will yield a DNA fragment of 1793 bp; If the cDNA insert is in the 3' to 5' orientation, the digests will yield a fragment of 355 bp.

transfection method (Graham and VanDereb, 1973). Control transfections were set up using wild type *AcNPV* DNA. The transfected *Sf9* cells were maintained in *Sf9* culture medium in 6-well plates (GIBCOL/BRL) and incubated at 27 ^{0}C.

3.3.7 Plaque purification of the positive recombinants

Four to seven days post-infection, the supernatants of transfected *Sf9* cells were harvested, then diluted by serial dilution to 10^{-12} with *Sf9* culture medium. The diluted supernatants were used to infect fresh *Sf9* cells. The *Sf9* cells were then overlaid with 0.5% agarose containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3indolyl β-D-galactoside) (Horwitz *et al.*, 1964). The plaques containing positive recombinants were identified by the appearance of intracellular occlusion bodies in the infected cells. In addition, recombinant baculovirus will be *lacZ*$^-$ due to the fact that *lacZ* locus is replaced by glucocerebrosidase cDNA. Therefore, the color of the plaques is white instead of blue in the presence of X-gal. Cells infected by wild type *AcUW1.lacZ* DNA (non-recombinant) give rise to blue colonies (Gruenwald and Heitz, 1993).

3.3.8 Virus amplification

For each expression study, 12 well-separated individual white plaques with the occlusion body positive phenotype were picked to infect fresh *Sf9* cells. Each individual plaque was transferred to a T-25 flask (GIBCOL/BRL) containing $2x10^6$ cells in 5 ml of *Sf9* culture medium for virus amplification. Seven days post infection, the *Sf9* cells and culture medium were harvested for enzymatic activity assay and virus titre determination.

3.3.9 Enzyme activity assay and protein concentration assay

The infected *Sf9* cells and culture medium were harvested by centrifuging at 1500x g for 10 min and the cell pellets were resuspended in distilled water. Cell homogenates

were prepared by freeze-thawing the cell suspension at -80 °C and 10 °C for 5 times. The protocol for the artificial substrate 4-methylumbelliferyl-β–D-glucopyranoside (4MUGP) assay was modified from a previous publication (Choy, 1984). In brief, 1 to 5 μl of cell homogenate or culture medium was added to 90 μl of reaction mixture which contained of 70 μl of 5 mM 4MUGP, 15 μl of 200 mM citrate buffer (pH 5.5) and 5 μl of 2% sodium taurocholate. The reaction mixtures were incubated in a 37 ^{0}C water bath for 1/2 hr. The reaction was stopped by the addition of 1.4 ml of 0.2 M glycine buffer, pH 10.5. The intensity of fluorescence in the enzyme reaction mixture was determined using a Sequoia-Turner fluorometer (Model 450; Abbott Diagnostics, Abbott Park, IL). The enzyme activity was calculated based on the intensity of fluorescence emitted by 4MU of a known concentration under identical conditions. Protein concentration was measured by the method of Bradford (1976), using crystalline bovine serum albumin (Sigma, grade V) as a standard.

3.3.10 Confirmation of recombinant baculovirus using the PCR method

2 ml of culture supernatant was centrifuged at 2,000x *g* for 15 min at 4 ^{0}C to remove *Sf9* cells, followed by a centrifugation at 10,000x *g* for 30 min to collect virus pellets. 500 μl of preheated CTAB buffer was added to the virus pellets, and the tube was incubated at 60 ^{0}C for 1hr. The genomic DNA from the recombinant virus was extracted using the standard chloroform extraction and ethanol precipitation procedure (Maniatis *et al.*, 1989). The virus DNA was used as template and two glucocerebrosidase cDNA specific primers (primer I and II) were used for PCR amplification.

3.3.11 Virus titre assay and amplification

The infected *Sf9* cell stocks that demonstrated the highest recombinant glucocerebrosidase activity were utilized for the virus titre assay. The virus stock solution was subjected to a 10-fold serial dilution and used to infect fresh *Sf9* cells in 96-well. Virus titres were determined and expressed as plaque-forming units (pfu) per milliliter (O'Reilly *et al.*, 1992).

If the titres of infected *Sf9* cells stocks were beyond the range of 10^{10} to 10^{14} pfu/ml, these stocks were further amplified by a second round of amplification. Fresh *Sf9* cells were inoculated with recombinant baculovirus stocks at MOI=1. The infected *Sf9* cells were incubated at 27 ^{0}C for seven days.

3.3.12 Purification of recombinant glucocerebrosidase using hydrophobic interaction chromatography

A Phenyl-5PW column (21.5x150 mm, Bio-Rad) was washed with 3 bed volume of degassed 0.85 M $(NH_4)_2SO_4$, 0.02 M Na_2HPO_4, pH 6.0. The enzyme extract was applied to the column at a flow rate of 2 ml/min through a Pharmacia P-500 high-performance pump. A total volume of 62 ml of running buffer was pumped thought the column. 160 ml of 100% to 0% decreasing linear gradient [0.85 M $(NH_4)_2SO_4$, 0.02 M Na_2HPO_4, pH 6.0] was then run through the column. At the end of the application, an increasing linear gradient of 0 to 3% sodium cholate solution (total volume 100 ml) was applied through the column. Fractions with high specific enzyme activity were pooled and concentrated using an Amicon Centreprep™ concentrator. The enzyme fraction was equilibrated with 1% sodium taurocholate, 5mM β-mecaptoethanol, and 50% ethylene glycol, and stored at -20 ^{0}C.

3.4 Results

As described in the Methods, the recombinant plasmid containing human glucocerebrosidase cDNA was selected, purified and subjected to endonuclease digestion with *XhoI/BamHI*. These two endonuclease digestion sites are present in the insert and vector respectively. The orientation of insert was confirmed by the sizes of digested DNA fragments. The result is shown in Figure 3.3. The expected pattern for the recombinant plasmid with insert in the 5'-3' orientation showed 2 bands at 1793 bp and 4416 bp. Those with insert in the 3'-5' orientation showed 2 bands at 355 bp and 5854 bp respectively.

Glucocerebrosidase cDNA was amplified from recombinant baculovirus (Figure 3.4). As shown, the presence of a DNA band of 1.7 Kbp, the expected size of full length glucocerebrosidase cDNA, was noted when amplified from recombinant virus containing the normal glucocerebrosidase allele, mutant 1366G allele, mutant 1604G allele, and a known mutant allele, 1226G. Similar results were noted when using a known Gaucher allele, 1448C, and a positive PCR control (cDNA/pAcUWl) as templates. Restriction analysis using various endonucleases that cleave known sites of glucocerebrosidase cDNA verified that the PCR amplified 1.7 Kbp DNA bands were the full-length glucocerebrosidase cDNA. There was no amplification when genomic DNA of uninfected *Sf9* cells or wild type baculovirus *AcNPV*-transfected cells was used as a template.

Virus titres of infected *Sf9* cells were found to range from 10^{-10} to 10^{-14} pfu/ml/plaque. Based on an enzyme activity assay using a normal control allele also, the glucocerebrosidase specific activity increased as titre increased within this range (data not shown). For the expression of each allele, 12 plaques were tested for the

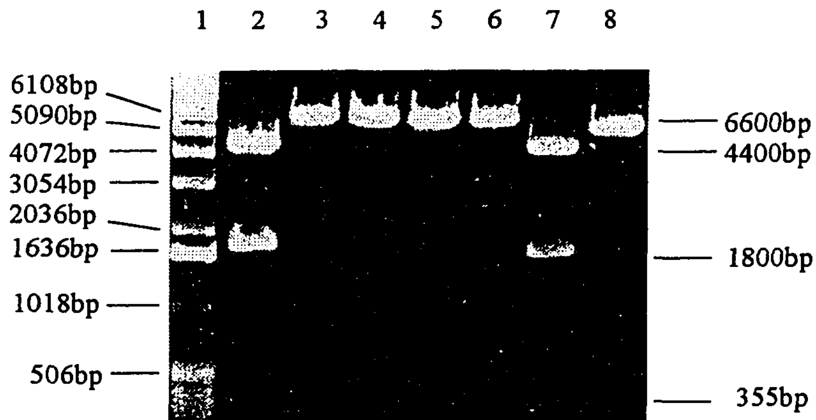


Figure 3.3 Identification of clones with glucocerebrosidase cDNA insert in the correct 5' to 3' orientation in the plasmid vector pAcUW1. Purified recombinant plasmid DNA was digested with *BamHI* and *XhoI* endonucleases and subjected for agarose gel electrophoresis. From the 7 clones analyzed, as shown from the left, it was noted that clones 1 (in lane 2) and 6 (in lane 7) have the glucocerebrosidase cDNA inserted in the correct orientation (Choy *et al.*, 1996).

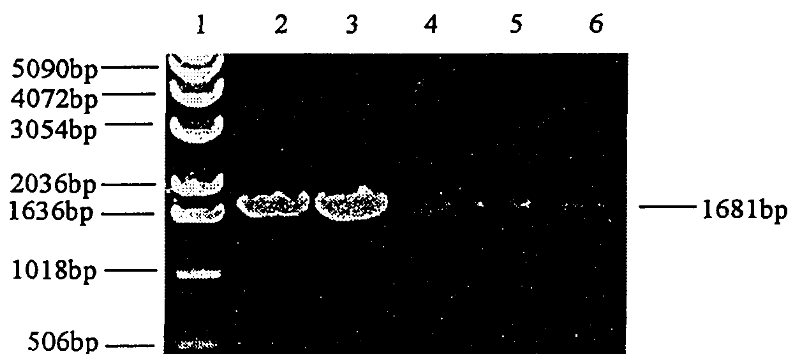


Figure 3.4 Demonstration of the integration of the full length glucocerebrosidase cDNA insert in the genomic DNA of the recombinant *Baculovirus*. PCR amplification was performed by using genomic DNA of the recombinant *Baculovirus* as a template and primers I and II flanking the entire coding region of glucocerebrosidase gene. The nucleotide sequences of these primers are shown in the text. From left: Lane 1, 1kb DNA ladder (GIBCO/BRL). Lanes 2 and 3, PCR control using purified recombinant plasmid DNA (GBA-cDNA/pAcUW1) as a template. Lanes 4, 5, and 6 are PCR products amplified from the gnomic DNA of the recombinant *Baculovirus* containing Gaucher l448C allele, 1366G allele, and a normal allele respectively (Choy *et al.*, 1996).

glucocerebrosidase activity. The recombinant plaque that exhibited the highest activity was selected. It was noted that recombinant baculovirus containing the normal allele and that containing 1226G allele have similar titres ($1.64x10^{10}$ for normal allele and $0.92x10^{10}$ for 1226G allele). The specific activity of the recombinant enzyme of normal allele was 4.3 hold higher than that of the 1226G allele (Table 3.1).

The levels of glucocerebrosidase activity determined in homogenates of baculovirus transfected *Sf9* cells are shown in Table 3.1. Enzyme-specific activity in transfected cells with the normal cDNA insert was 352.0 nmol/hr/mg protein. For comparison, the specific activities of glucocerebrosidase from leukocytes of normal and patient BD were 7.8 nmol/hr/mg and 0.88 nmol/hr/mg. This indicates that the specific activity of the normal recombinant glucocerebrosidase is more than 45x higher than that in normal leukocytes.

Enzyme specific activity in transfected cells with mutations 649T, 1366G, 481T, and 1604A alleles are 2.8%, 2.9%, 17.3% and 6.9% that of the normal allele, respectively. By comparison, the enzyme specific activity in transfected cells of another known Gaucher allele, 1448C, prevalent in type 2 Gaucher disease, was 3.3% of normal. No endogenous enzyme activity could be detected in the control experiment in *Sf9* cells transfected with *AcNPV* (Table 3.1).

The extract of cell homogenates from infected *Sf9* cells, which expressed normal glucocerebrosidase, was applied to a Phenyl hydrophobic interaction column for enzyme purification. The recombinant enzyme remained bound to the column during the elution with the desalting gradient, and was only eluted at a cholate concentration of 1% during cholate gradient elution (Figure 3.5). The eluted fractions from the Phenyl-5PW

Glucocerebrosidase alleles	Enzyme activity[a] (nmol/hr/mg protein)	%
Normal	352	100
Null[b]	0	0
Mutation 649T	9.7	2.8
Mutation 1366G	10.1	2.9
Mutation 481T	61.0	17.3
Mutation 1604A	24.3	6.9
Mutation 1226G	82.1	23.1
Mutation 1448C	11.7	3.3

[a] The level of glucocerebrosidase activity shown is the average of a triplicate assay that varies by less than 15%
[b] This is the control experiment using *Sf9* cells transfected by wild type *Baculovirus AcNPV*.

Table 3.1 Glucocerebrosidase specific activity from transfected *Sf9* cell homogenates.

chromatography with high enzyme specific activities were pooled and concentrated using Amicon Centreprep™ concentrators. The recombinant enzyme was found to retain catalytic activity on 4MUGP substrate after purification. The specific activity of the normal recombinant glucocerebrosidase increased from 67.8 to 3836.1 nmol/hr/mg after a single step of chromatography, which represented more than 56-fold purification. The yield was 80.9 %. Similar results were obtained in other separate experiments.

3.5 Discussion

The baculovirus expression system has been shown to be effective in the expression of recombinant glucocerebrosidase (Martin *et al.*, 1988; Grabowski *et al.*, 1989; Grace *et al.*, 1994). However, it was necessary to test the reliability of our expression system and to compare the levels of glucocerebrosidase activity expressed by the 649T, 1366G, 481T and 1604A alleles. We cloned Gaucher allele 1226G into baculovirus *AcUW1.lacZ* for transfection and expression studies. This was also done for a second known mutant allele 1448C, frequent in neuronopathic Gaucher with a poor prognosis (Tsuji *et al.*, 1987). The specific activities of glucocerebrosidase determined in transfected *Sf9* cells with mutations 1226G and 1448C were 23.1% and 3.3% of the control, respectively (Table 3.1). This finding demonstrates the reliability of the baculovirus expression system in transfected *Sf9* cells for the expression and testing of Gaucher alleles. Our finding further demonstrated that the baculovirus expression system in insect cells appears to be a suitable system for expression studies due to a lack of endogenous glucocerebrosidase activity in either uninfected or transfected *Sf9* cells without the glucocerebrosidase cDNA insert. This advantage has permitted us to detect

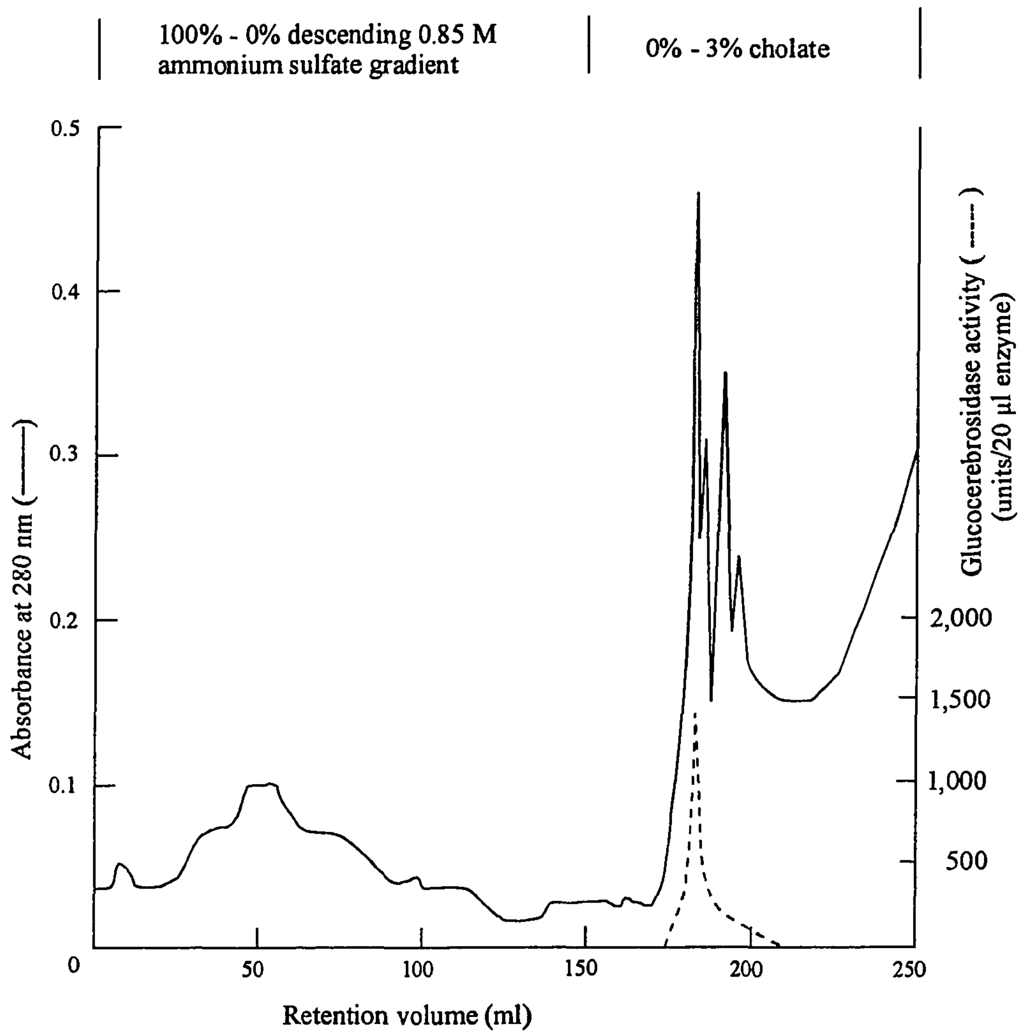


Figure 3.5 Phenyl-5PW (Bio-Rad) hydrophobic interaction chromatography of recombinant glucocerebrosidase. The enzyme activity was determined using 4MUGP as a substrate.

the expression of very low enzyme activity by some Gaucher alleles, such as the mutant 1366G allele (Table 3.1)

The expression studies of mutant alleles 649T, 1366G, 481T, and 1604A indicated that they are deleterious and not neutral mutations as their expression results in profoundly deficient enzyme activity (Table 3.1). Although mutation 481T results in Pro to Ser substitution at amino acid residue 122, which may result in the conformation change of glucocerebrosidase structure, the enzyme activity of expressed mutant alleles 481T was relatively high (17.3% of normal). It was also noted that the enzyme activity of expressed mutant allele 649T is extremely low. It has only about 3% of normal activity. Based on the observation that there was severe neurological involvement in patient BD, and that Gaucher patients with other genotypes such as the 1226G/1448C have non-neuronopathic and sometimes relatively mild clinical course, we postulate that mutation 649T may result in a poor prognosis in Gaucher disease. The levels of residual glucocerebrosidase activity expressed by the known Gaucher alleles 1226G and 1448C appear to correlate with the severity associated with Gaucher type 1 and type 2 clinical forms (Table 3.1). Although it is tempting to speculate that such a correlation exists, this remains a speculation until the normal and mutant enzymes can be purified, characterized, and compared.

From the gene expression studies, it appears that there may be some correlation between genotypes and clinical phenotypes. Mutation 1226G is postulated to result in the non-neuronopathic type 1 form of Gaucher disease and, when homozygous, is associated with a good prognosis (Zimran *et al.*, 1989; Sibille *et al.*, 1993). Although mutations 1604A and 1366G in patients ES and BL, respectively, have only 6.9% and 2.9% of specific enzyme activity in the baculovirus expression system, Both patient ES and BL

appears to have type 1 Gaucher disease without neurological complications. This may be due to the mutant allele (1226G) which presents as a heterozygous form in both patients. It will be interesting to screen for the presence of the 1366G and 1604A alleles in different heterozygous genotypes (e.g., 1366G/1448C, and 1604A/1448C) or homozygous genotypes (e.g., 1366G/1366G, and 1604A/1604A) among Gaucher patients and compare the clinical phenotypes between those with that of patients ES and BL.

Chapter 4 Cloning, Expression, and Characterization of Human Glucocerebrosidase in the *Pichia Pastoris* Expression System

4.1 Abstract

As a subtype of an inherited lysosomal storage disorder, type 1 Gaucher disease can be effectively treated by enzyme replacement therapy. Therefore, the evaluation and development of an effective method for large scale production of recombinant glucocerebrosidase will have a significant potential for therapeutic applications.

Glucocerebrosidase cDNA was cloned into transformation vectors pPIC9K and pPICαZ downstream from the *AOX1* promoter, and integrated into yeast hosts KM71 and SMD1168 of *Pichia pastoris*. When the native targeting signal of glucocerebrosidase cDNA was replaced with an α-factor secretion signal, the recombinant glucocerebrosidase was expressed and secreted into the culture medium. The maximum expression level under flask culture conditions reached a specific activity of 494 nmol/hr/mg protein on the glucocerebroside substrate. The size of the secreted form of recombinant glucocerebrosidase was determined to be 66 KDa. After deglycosylation, the polypeptide backbone has a molecular weight of 58 kDa. The recombinant enzyme reacts to monoclonal antibodies directed against native glucocerebrosidase or a short peptide of the enzyme. The recombinant enzyme exhibits kinetic properties similar to that of native glucocerebrosidase. A successive two-step chromatography procedure was also developed to purify the recombinant enzyme to apparent homogeneity.

This is the first description of heterologous expression of glucocerebrosidase in the yeast *P. pastoris*. Our study demonstrated that functionally active glucocerebrosidase can be expressed and secreted excellularly using the *Pichia* expression system.

4.2 Introduction

Enzyme replacement therapy is one of the effective treatments for type 1 Gaucher disease (Barranger and Ginns, 1989; Grabowski *et al.*, 1995; Brady and Barton, 1996). It has been demonstrated that intravenous infusion of exogenous glucocerebrosidase can reduce the level of accumulated glucocerebroside (Brady and Barton, 1996). Several methods for purification of native placental glucocerebrosidase have been developed (Grabowski and Dagan, 1984; Osiecki-Newman *et al.*, 1986; Choy, 1986; Aerts *et al.*, 1986). Due to the limited supply of natural source of this enzyme, effort has focused on the production of recombinant glucocerebrosidase. Recombinant glucocerebrosidase has been reportedly expressed in several different expression systems, such as NIH3T3 (mouse fibroblast) cells (Sorge *et al.*, 1987), invertebrate *Spodoptera frugiperda* (*Sf9*) cells (Martin *et al.*, 1988; Grabowski *et al.*, 1989) and mammalian COS-I cells (Grabowski *et al.*, 1989). Macrophage-targeted placental glucocerebrosidase and a recombinant glucocerebrosidase produced from Chinese hamster ovary cells have been used for several years in the treatment of Gaucher disease with success (Grabowski *et al.*, 1995; Brady and Barton, 1996). However, enzyme replacement therapy is prohibitively expensive (Garber, 1992). It is therefore desirable to identify and utilize an alternative expression system that can be used for mass production of active recombinant glucocerebrosidase for enzyme replacement therapy of Gaucher disease.

P. pastoris, a methylotrophic yeast, has been found to be efficient in expressing heterologous intracellular or secreted proteins (Cregg *et al.*, 1993). It is capable of growing on methanol as a sole carbon and energy source. Its methanol-inducible alcohol oxidase (*AOX*) is the first enzyme in the methanol utilization pathway which catalyzes the

oxidation of methanol to formaldehyde (Anthony, 1982). There are two *AOX* genes (*AOX1* and *AOX2*) in *P. pastoris*. *AOX1* is responsible for all but a minor fraction of total *AOX* message and protein (Cregg *et al.*, 1993). The *AOX1* promoter can drive heterologous gene expression to high levels (Cregg *et al.*, 1993).

Several recombinant mammalian proteins have been expressed using the *Pichia* expression system (Invitrogen, 1994; Figure 4.1). Since glucocerebrosidase is a glycoprotein (Takasaki *et al.*, 1984), glycosylation properties are a major consideration in choosing expression systems. It has also been demonstrated that glycosylated proteins expressed in *P. pastoris* do not have 1,3 glycan linkages, which are responsible for the hyper-antigenic nature of proteins secreted from *Saccharomyces cerevisiae* (Cregg *et al.*, 1993). The majority of N-linked oligosaccharide chains on proteins secreted from *P. pastoris* are the high-mannose type, with much shorter glycans than those of *S. cerevisiae* (Cregg *et al.*, 1993; Miele *et al.*, 1997). These features make *P. pastoris* very attractive for the expression of recombinant glucocerebrosidase for therapeutic purposes. In addition, mannose-terminal glycoconjugates promote interaction with lectins on the surface of macrophages (Brady and Barton, 1996). Therefore, we predict that the mannose-terminal enzyme will be more effectively targeted to macrophages than native glucocerebrosidase. Thus, sequential deglycosylation by enzymatic or chemical approaches to remove non-mannose cabohydrates of glycan side chains may not be necessary.

To evaluate this microbial eukaryotic expression system for its ability to produce functional recombinant glucocerebrosidase, we cloned the full length of the glucocerebrosidase cDNA in the *Pichia* expression vectors, and transformed them into *P. pastoris* strains KM71 and SMD1168. The recombinant clones that expressed and

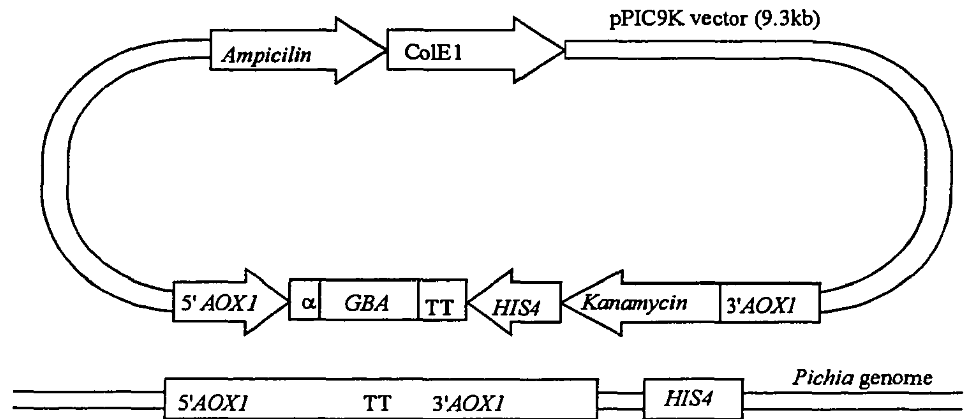


Figure 4.1 The *Pichia pastoris* expression system. Gene integration can occur by a single crossover between the *AOX1* locus in the *Pichia* genome and any of the three regions on the vector, or by a double crossover event between the *AOX1* promoter and 3' *AOX1* regions of the vector and *Pichia* genome (Invitrogene, 1994b).

secreted glucocerebrosidase into the inducing medium were selected from the KM71 strain, which was transformed with recombinant vector pPICαZ. The recombinant enzyme was purified to apparent homogeneity using a two-steps chromatography procedure. The purified recombinant glucocerebrosidase was found to maintain catalytic activity on the natural substrate and artificial substrate, and have similar kinetic properties to that of the native enzyme.

4.3 Materials and methods

4.3.1 Materials

The following were from commercial sources. Conditurol-B-epoxide, sodium cholate, N-palmitoyl-DL-dihydro-glucocerebroside, bovine serum albumin, Triton X-100, 4-methylumbelliferyl-β-D-glucopyranoside (4MUGP), peptide N-glycosidase F (Sigma, St. Louis, MO); RPMI 1640, fetal bovine serum, trypsin, penicillin, streptomycin, *Taq* DNA polymerase (GIBCO/BRL, Grand Island, NY); sodium taurocholate (Cal Biochem, La Jolla, CA); trisodium citrate, citric acid (Caledon Laboratories, Georgetown, ON); Restriction endonucleases (New England Biolabs, Beverly, MA); ammonium sulfate (BDH, Toronto, ON); ethylene glycol (Anachemia, Rosese Point, NY); Peptone, yeast extract and yeast nitrogen base (Difco, Detroit, MI); *Pfu* DNA polymerase (Strategene, La Jolla, CA); monoclonal anti-glucocerebrosidase antibody (14B4) was a gift from Dr. Beutler (The Scripps Research Institute, La Jolla, CA).

4.3.2 *Escherichia coli* and *Pichia pastoris* strains

E. coli strain Top 10F' (Invitrogen, San Diego, CA.) was used to generate recombinant vectors. *P. pastoris* strains KM71 and SMD1168 (Invitrogen, San Diego, CA.) were used as hosts for expression studies. Both yeast host strains have a defect in the

histidinol dehydrogenase gene (*HIS4*⁻). KM71 has a defect alcohol oxidase gene (*AOX1*⁻), and SMD1168 bears a protease deficiency (*PEP4*⁻).

4.3.3 Generation of the monoclonal antibody against glucocerebrosidase

A short peptide containing 17 amino acid residues of glucocerebrosidase (amino acid residues 99-115: PAQNLLLKSYFSEEGIG) was synthesized by Research Genetics (Huntsville, AL). Eight copies of the peptide were conjugated onto a branched lysine core to form an antigen. Four amino acid residues in this short peptide are different between human and mouse. The amino acid residues from position 99-115 in mouse are PTQKLLLKSYFSTEGIE.

BALB/c mice were primarily immunized by interaperitoneal injection with 50 µl antigen (2 µg/µl) emulsified in an equal volume of Freund's complete adjuvant (GIBCO/BRL, Grand Island, NY), followed by three boost injections with 50 µl antigen and Freund's incomplete adjuvant, each at 3-week intervals. The antiserum of immunized mice was tested for immuno-responses using the dot blot method (Suresh and Milstein, 1985). The spleen cells of immunized mice were fused with myeloma cells. Hybridoma was selected on Clona™-HY medium (Stemcells, Vancouver, BC). More than 200 hundred monoclonal colonies were further amplified, and screened for the positive antibody. Several positive antibody clones were obtained. Some of those clones were used to produce ascitic fluid.

4.3.4 Fibroblast cultures, mRNA isolation and cDNA synthesis

The experimental details of the above procedures were described in Chapter 2.

4.3.5 Construction of recombinant vectors

The plasmids pPIC9K (Invitrogen, 1994a) and pPICZα (Invitrogen, 1994b) were used as yeast transformation vectors. Both vectors carry *AOX1* 5' and 3' fragments for homologous integration at this locus, as well as the *AOX1* promoter and an α-factor secretion signal. Glucocerebrosidase cDNA was PCR-amplified using *Pfu* DNA polymerase that possesses proofreading capability to ensure high fidelity. The cDNA was then cloned into the vectors at the *EcoR*I site, downstream from the *AOXI* promoter and α-factor secretion signal, and upstream from the *AOX1* transcriptional terminating sequence. Different constructions were generated for this study (Figure 4.2).

i). Primer 1 (5'-TCACGAATTCTCATCTAATGACCCTGAGG-3') and primer 2 (5'-GCGGGAATTCTTTAATGCCCAGGCTGAGCC-3') were used to generate the full length of glucocerebrosidase cDNA (1680 bp; including native targeting signal and mature protein) containing *EcoR*I restriction sites at the 5' and 3' of the sequence. The insert was cloned into the vector pPIC9K to construct the recombinant vector α-NTS-MP/pPIC9K (NTS represents native targeting signal; MP represents mature protein).

ii). Primer 3 (5'-TATGAATTCGCCCGCCCCTGCATCCCT-3') and primer 4 (5'-GTCAGCTAGCTGGCGACGCCACAGTA-3'); containing an *Nhe*I digestion site in its 5' end) were used to generate the glucocerebrosidase cDNA (1517 bp) without native target signal, but containing sequences for a hexahistidine and C-myc epitope tag (peptide tag: PT) at its 3' end. The insert was double-digested with *EcoR*I and *Nhe*I, and cloned into the vector pPICZα (*EcoR*I and *Xba*I digested) to construct the recombinant vector α-MP-PT/pPICZα.

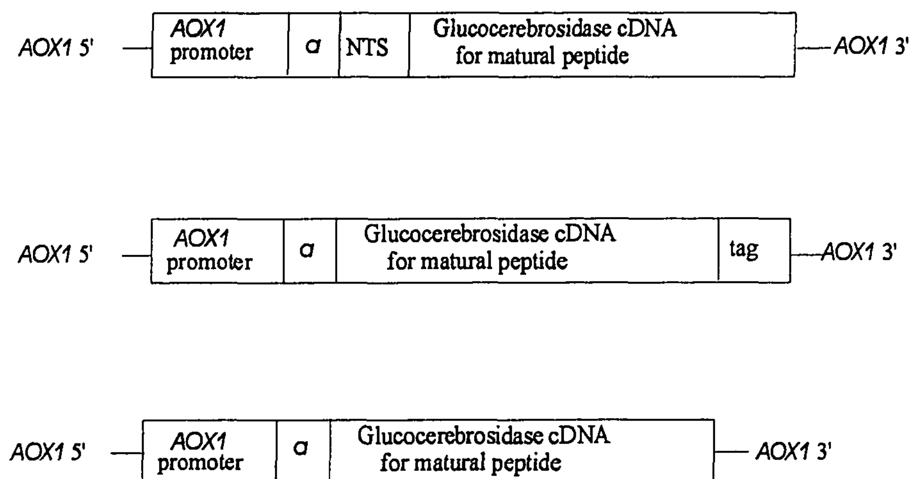


Figure 4.2 Constructs of recombinant vectors.α is an α-factor secretion signal; NTS is a glucocerebrosidase native targeting signal; Tag is a hexahistidine and a myc epitope.

iii). Primer 2 and primer 3 were used to generate the glucocerebrosidase cDNA (1560bp) without native target signal and without the hexahistidine and C-myc epitope tag at its 3'. The insert was cloned into the vector pPICZα at the *EcoR*I site to construct the recombinant vector α-MP/pPICZα.

The recombinant vectors were transferred into *E. coli* Top10F' cells. The orientation of the insert was confirmed by digesting the purified recombinant vectors with two unique restriction enzymes (*BamH*I and *Xho*I) which are present in the insert and vector, respectively. The recombinant plasmid was also sequenced to confirm that the glucocerebrosidase codons were in frame with the *AOX1* promoter and α-secretion signal sequence using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with the fmol™ DNA sequence kit (Promega, Madison WI).

4.3.6 Yeast transformation and clone selection

The purified recombinant vector α-NTS-MP/pPIC9K with the correct insert orientation was linearized with *Bgl*II, and transformed into the *P. pastoris* genome using the spheroplasting method (Invitrogen, 1994). That of α- MP-T/pPICZα and α-MP/pPICZα were transformed using the Easycomp™ yeast transformation kit (Invitrogen, San Diego, CA). *His4*$^+$ transformants were collected from the transformation plates, resuspended in distilled water and diluted to $5x10^5$ cells/ml. 200 μl of suspension solutions of α-NTS-MP/pPIC9K transformants were spread onto each YPD (1% yeast extract, 2% bacto-peptone, 2% dextrose and 2% agar) plate containing G418 (Geneticin) (GIBCLO/BRL, Grand Island, NY) at the concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 mg/ml, and that of α- MP-T/pPICZα and α-MP/pPICZα were spread onto

YPD plates, but containing Zeocin instead of G418. Zeocin resistance clones were then placed on MM (minimal methanol plate: 1.34% yeast nitrogen base, 4x 10^{-5}% biotin, 0.5% methanol, 1.5% agar) and MD (minimal dextrose plate: 1.34% yeast nitrogen base, 4x 10^{-5}% biotin, 1% dextrose, 1.5% agar) plates for screening for Mut^{+} (wild type for methanol utilization) and Mut^{s} (methanol utilization slow) transformants. In brief, each single clone was picked and streaked on both MM and MD plates, and incubate for 2 days at 30 ^{0}C. If *AOX1* gene was disrupted, the clone would grow poorly on MM plates, showing Mut^{s} phenotype.

4.3.7 Confirmation of gene integration

i). PCR confirmation. The genomic DNA of selected recombinant yeast clones (as well as control clones) was extracted using Zymolase (Seikagaku America, Rockville, MA) and DNAzol reagent (GIBCO/BRL, Bethesda, MD). In brief, the recombinant yeast clones were cultured in YPD liquid medium for two days at 30^{0}C with shaking. Cells from 1.5 ml of culture mixture were harvested by centrifuging at 3,000x *g* for 5 min, and cell pellets were treated with 15 µl Zymolase (0.3 U/µl in water) by incubating the mixture at 37 ^{0}C for 1hr. DNAzol was added to the preparations for genomic DNA extraction following the manufacture's protocol. Primers 3 and 4 (as described in section 4.3.5) were used to amplify the glucocerebrosidase cDNA fragments from the recombinant *P. pastoris* genome.

ii). Southern blotting. Equal amounts (10 µg) of extracted yeast genomic DNAs were digested with *EcoR*I to cleave the glucocerebrosidase cDNA from the *Pichia* genome. The *EcoR*I- digested samples were run on a 0.7% agrose gel, denatured with 0.25M NaOH, and then transferred to a Hybond-N Nylon membrane (Amersham Life

Science, Toronto, ON) using the Mini Trans-Blot Electrophoretic transfer (Bio-Rad, Hercules, CA). The membrane was then hybridized with a radioactive (α^{32}-P dATP) probe (300bp) of glucocerebrosidase cDNA, which was prepared using the method of Rashichian (1994). The membrane was exposed to UV light (320 nm) for 1.5 min to cross link the DNA to the nylon. Autoradiography of the membrane was carried out using BioMax™ film (Kodak, Rochester, NY) exposed for 24 hr at room temperature.

4.3.8 Expression of recombinant *Pichia* clones

Expression of recombinant glucocerebrosidase was conducted according to the protocol provided by Invitrogen (San Diego, CA) with some modification. In brief, single colonies of transformants were first inoculated in 5x150 ml of BMGY medium (1% yeast extract, 1% casamino acids, 2% bacto-peptone, 0.67% yeast nitrogen base without amino acids, $4x10^{-5}$% biotin, 1% glycerol, 0.1 M citrate buffer, pH5.0), in 500 ml baffled flasks. The baffled flasks were shaken at 250x RPM in a shaking incubator for 48 hr at 30 ^{0}C. placed. After centrifugation (10 min, 2,500x *g*), cells were resuspended in 4 x 50 ml of BMMY medium (with glycerol replaced by 0.5% methanol as the sole source of carbon and energy) for three days. At 24 hr intervals, the yeast cultures were fed with methanol at 0.5% of culture volume, and samples of the expression cultures were taken to analyze expression levels of the recombinant glucocerebrosidase. During the first 24 hr induction period, the pH of the medium was found to increase from 5 to 7.5. Citrate buffer (pH 4.0) was added to adjust the pH back to 5.0. After 72 hr of methanol induction, the culture medium and yeast cells were separated by spinning at 2,500x *g* for 10 min. The two fractions were separated and stored at -80 ^{0}C until needed.

4.3.9 Northern blotting

Selected recombinant yeast clones were inoculated in BMGY and induced with methanol as described in the previous paragraph. Cell pellets were harvested during the induction period at 0, 24, 48, 72, 96 hr intervals. The cell pellets were stored at -80 ^{0}C until use. The total RNA was isolated using the hot phenol method (Ausubel *et al.*, 1995). 10 μg of each RNA was run on a 1% formadehyde-agarose gel and transferred to a Hybond-N Nylon membrane (Amersham Life Science, Toronto, ON) by capillary blotting. After exposure to UV light (320 nm) for 1.5 min, the membrane was subjected for hybridization and autoradiography as described for Southern blotting.

4.3.10 Recombinant enzyme preparation

The cell pellets were washed, resuspended in ice-cold breaking buffer (50mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 5% glycerol), and vortexed with an equal volume of glassbeads (Sigma, St. Louis, MO) at 4 ^{0}C. To solubilize membrane-bound enzymes, 20% sodium taurocholate was added to cell homogenates to a final concentration of 1%, and the mixtures were put on ice and shaken gently for 1 hr. The mixtures were spun at 14,000x *g* for 15 min at 4 ^{0}C, and cell lysate was collected for the analysis.

4.3.11 Enzymatic activity assay and protein concentration assay

For the natural substrate (N-palmitoyl-DL-dihydro-glucocerebroside) assay, the activities of recombinant glucocerebrosidase from culture medium and cell pellets were measured as previously described (Choy, 1989). In brief, 10 to 50 μl of enzyme preparation or culture medium was added to 100 μl of dispersed mixtures consisting of 1 mM N-palmitoyl-DL-dihydro-glucocerebroside, 1% sodium taurocholate, 0.1% Triton X-

100, and 40 mM citrate buffer, pH 5.5. The reaction mixtures were incubated in a 37 ^{0}C shaking water bath for 4 hr. The reaction was stopped by immersing the tubes in boiling water for 5 min. The reaction mixtures were then centrifuged at 14,000x *g* for 15 min at 4 ^{0}C. 90 μl of supernatant was transferred to a tube that contained 540 μl of glucose reagent (Sigma, St. Louis, MO). After 5 min incubation at room temperature, the glucose reagent mixtures were measured at 340 nm UV using a Spectronic Senesys5^{TM} spectrophotometer (Milton Roy, Rochester, NY). The enzyme activity was calculated based on the amount of glucose present with respect to a glucose standard curve (0-60nmol). To subtract endogenous glucose, a parallel test was done under identical conditions without the natural substrate.

The protocol for the artificial substrate 4MUGP assay was modified from a previous publication (Choy, and Davison, 1980). 0.5 to 10 μl of enzyme preparation was added to 90 μl of the reaction mixture which comprised of 70 μl of 5 mM 4MUGP, 15 μl of 200 mM citrate buffer (pH 5.5) and 5 μl of 2% sodium taurocholate. The reaction mixtures were incubated in a 37 ^{0}C water bath for 1/2 hr. The reaction was stopped by the addition of 1.4 ml of 0.2 M glycine buffer, pH 10.5. The intensity of fluorescence in the enzyme reaction mixture was determined using a Sequoia-Turner^R fluorometer (Model 450; Abbott Diagnostics, Abbott Park, IL). The enzyme activity on 4MUGP was calculated based on the intensity of fluorescence emitted by 4MU of a known concentration under identical conditions. Protein concentration was measured by the method of Bradford (1976), using crystalline bovine serum albumin (Sigma, grade V) as a standard.

4.3.12 Kinetic assay

The apparent Michaelis constant (K_m) was determined using Lineweaver-Burk plot according the method of Choy and Davidson (1980a). The concentrations of substrate (4MUGP) in the kinetic study were 0.33, 0.4, 0.5, 0.67, 1, 2, 3, 4, and 5 mM, respectively.

4.3.13 pH profile assay

The pH profile assay was done as described for the activity assay for 4MUGP substrate, except the pH of citrate buffer in the reaction mixture were prepared as pH 3.5 to pH 8 at 0.5 pH intervals.

4.3.14 Inhibitor assay using conduritol B-epoxide (CBE)

50 μl yeast culture medium that contained recombinant glucocerebrosidase was mixed with 20 μl of 10 μM CBE and kept on ice for 1 hr. The treated and control samples were then assayed for the activities on the natural substrate using the procedure described previously.

For the IC_{50} assay, partially purified enzyme and CBE mixtures were prepared with concentrations of CBE of 0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, and 5 μM. The mixtures were incubated on ice for 1hr and subjected for enzyme activity assay using fluorogenic substrate 4MUGP.

4.3.15 Endoglycosidase deglycosylation

Recombinant glucocerebrosidase from the yeast cell homogenates was denatured with 0.1% SDS in the presence of 0.1% β-mercaptoethanol. The enzyme preparation was then added to the reaction mixture containing 0.8 units of peptide N-glycosidase F (Boehringer Mannhein, Indiannapolis, IN), 0.1% β-mercaptoethanol, 0.5% Triton-X, 25

mM EDTA, and 150 mM sodium phosphate, pH 7.5. The reaction was incubated at 37 ^{0}C for 18 hr, followed by SDS-PAGE separation and Western blot detection.

For deglycosylation of the purified recombinant enzyme, the enzyme preparation was diluted with 100X volume of the following solution: 20 mM citrate buffer, pH 6.0, 100 mM NaCl, 5 mM β-mercaptoethanol, and then concentrated to the original volume using an Amicon Centreprep™ concentrator, which was pre-treated with 1% BSA overnight at 4 ^{0}C to minimizing non-specific adsorption by the concentrator membrane. Glycerol was then added to the dialyzed enzyme preparation to a final concentration of 30%. One unit of peptide N-glycosidase F (Sigma, St. Louis, MO) was added to the mixture and incubated at 37 ^{0}C for 48 hr. The reaction was subjected to a SDS-PAGE separation and silver staining detection.

4.3.16 Protein silver staining and Western blotting

Protein samples were separated by SDS-PAGE (Laemmli, 1970) in a 10% gel. For silver staining, the gel was fixed and developed as described by the Bio-Rad Silver staining kit (Hercules, CA). For Western blot, the protein samples were electro-blotted to nitrocellulose membranes (Hitroplus 2000, MSC, Westbord, MA) using a mini electrophoretic transblot apparatus (Bio-Rad, Hercules, CA). Immunoblots were developed by incubation with the glucocerebrosidase monoclonal antibody (1:200), followed by an anti-rabbit IgG conjugated to horseradish peroxidase. The antigen-antibody complex was detected using Renaissance® enhanced luminol chemiluminescence regent (NEN™ Life Sciences, Boston, MA).

4.3.17 Purification (FPLC)

The recombinant yeast clone K4-13 was used for large scale flask culture. After 72 hr of induction, the culture medium (BMMY, pH 5.0) was harvested and stored at -80 ^{0}C until use. Phamacia's LCC-500 FPLC system was used for the purification of recombinant glucocerebrosidase. All chromatographic runs were carried out at 4 ^{0}C.

Hydrophobic chromatography: The culture medium of K4-13 was equilibrated with $(NH_4)_2SO_4$ to 0.85 M, and centrifuged at 12,000x *g* for 15 min at 4 ^{0}C. It was then applied to a Phamacia Phenyl-Sepharose column (150 x 21.5 mm) at flow rate of 2 ml/min. The proteins adsorbed to the column were eluted at a flow rate of 2 ml/min, using 120 ml of a 100% to 0% decreasing linear gradient [0.85 M $(NH_4)_2SO_4$ and 20 mM Na_2HPO_4, pH 7.0], and followed by 120 ml of ddH_2O. The column was then eluted with 120 ml of 0 to 100% increasing linear gradient 2% sodium cholate. The eluted fractions were collected using Phamarcia FRAC-100 fraction collector. The eluted fractions with high enzyme specific activities during ddH_2O elution period and during the sodium cholate elution period were pooled separately. A solution of 100 mM β-mercaptoethanol was added to the elutants to a final concentration of 5 mM. The samples were stored at 4 ^{0}C.

Ion-exchange chromatography: The enzyme preparation from the phenyl column purification (ddH_2O fractions) was diluted with an equal volume of 20 mM Bis-Tris (pH 7.0), and applied to a Q-Sepharose column (Phamacia, 30 x 10 mm), which was pre-euuilibrated with 1 M NaCl in 20 mM Bis-Tris (pH 7.0). The proteins absorbed to the column were eluted at flow rate of 0.5 ml/min, using 60 ml of 0 to 100% increasing linear gradient 1M NaCl and 20 mM Bis-Tris, pH 7.0, followed by 45 ml of the same elution buffer. The eluted fractions with high enzyme specific activities were pooled, and 100 mM

β-mercaptoethanol added to a final concentration of 5 mM, 2% sodium taurocholate to a final 0.1% and 500 mM citrate buffer (pH 4.0) to a final concentration of 15 mM. The eluent was then concentrated using an Amicon Centreprep™ concentrator, which was pre-blocked with 1% BSA. After the concentration, an equal volume of ethylene glycol was added to the enzyme preparation. The sample was stored at -20 ^{0}C.

4.4 Results

4.4.1 Gene integration and RNA transcription

Transformation efficiency. A transformation efficiency of 596 to 778 transformants per μg of DNA was obtained using the electroporation method for the recombinant construct α-NTS-MP/pPIC9K. Although the transformation efficiency was lower when using a spheroplasting method (389 to 535 transformants per μg of DNA), the percentage of transformants that contained higher copy numbers of inserts was increased (data not shown). Since most of the transformants grew well on MM and MD plates, This Mut$^+$ (Methanol utilization plus) phenotype indicated that most of the gene integration events occurred via single crossovers between the homologous sequences in the recombinant constructs and the *Pichia* genome, resulting from insertion of the expression cassette in the *Pichia* genome without displacement of the native *AOX1* gene (Invitrogen, 1994).

PCR confirmation. The full length of glucocerebrosidase cDNA was amplified by PCR using recombinant *P. pastoris* genomic DNA as a template (Figure 4.3). No DNA fragment was amplified when using genomic DNA of a control yeast clone (transformed with vector only) as a template. This indicated that glucocerebrosidase cDNA was integrated into the *P. pastoris* genome.

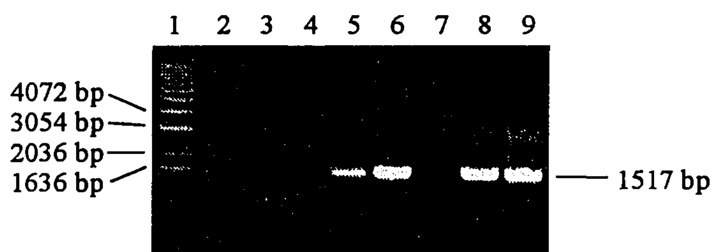


Figure 4.3 PCR confirmation of glucocerebrosidase gene integration in the genome of *Pichia pastoris*. PCR amplification was performed using genomic DNA of the recombinant *P. pastoris* as a template, and primers 3 and 4 flanking the entire coding region of glucocerebrosidase cDNA. The nucleotide sequences of these primers are shown in the text. From left: Lane 1, 1kb DNA ladder (GIBCO/BRL). Lanes 2, PCR negative control. Lane 3 and 4, recombinant control, yeast clones containing vector only. Lane 5 - 8, various recombinant clones. Lane 9, PCR positive control.

Selection of multiple copy integration and Southern blotting analysis. For the selection of multiple copy insertion of the constructs, it was observed that as the concentration of G418 increased, fewer colonies grew on each plate. There were only 2 to 4 colonies that grew on YPD plates containing 3-4 mg/ml G418, whereas on the control plate, YPD containing no G418, had over 1000 colonies. Those transformants that survived on the high concentration of G418 indicated that they contained several integrated copies of the expression cassettes, since a high copy number of G418 resistance gene inserts is required for the transformants to grow on the high concentration G418 media (Invitrogen, 1994b; Scorer *et al.*, 1994). The results of Southern blotting analysis showed that the band intensity of hybridization increased when DNA of transformants grown in a high concentration of G418 was used (data not shown).

A similar approach was used to select multiple copy inserts when using pPICZα vector and antibiotic Zeocin. It was noted that colony number on each Zeocin plates decreased when the concentration of Zeocin increased from 0.25 to 2 mg/ml; but there was no difference on colony numbers when the concentration of Zeocin increased from 2 to 4 mg/ml.

Northern blotting analysis. Examination of RNA accumulation, by Northern blotting analysis, in induced recombinant *P. pastoris* cells showed that glucocerebrosidase cDNA under the control of the *AOX1* promoter was transcribed at the anticipated size (Figure 4.4). A single band of 2.2 kb (glucocerebrosidase cDNA plus flanking sequences) was detected from total RNA extracts of recombinant *P. pastoris* at 24, 48, 72 and 96 hr post-induction with methanol. No glucocerebrosidase mRNA was observed at 0 hr post-

induction, or from the control clone (recombinant *P. pastoris* containing pPICZα vector only).

4.4.2 Expression of three glucocerebrosidase constructs

In the specific enzyme activity assay of both medium and cell pellet fractions of α-NTS-MP/pPIC9K-containing recombinants, no functionally active recombinant glucocerebrosidase was detected using natural lipid substrate assay. The Western blotting analysis of cell pellet fractions revealed that the anti- glucocerebrosidase monoclonal antibody reacted to proteins of 90, 75, and 52 kDa (Figure 4.5); No bands were detected on the control clone which was transformed with vector only.

The expression of α-MP-T/pPICZα. and α-MP/pPICZα. Since no functional activity was detected from the transformants of α-NTS-MP/pPIC9K, we re-cloned the glucocerebrosidase cDNA containing the sequences for mature protein only into the vector pPICZα downstream from α-secretion factor, and transformed the recombinant constructs into KM71 and SMD1168 of *P. Pastoris*. The functional catalytic activity of glucocerebrosidase was detected from the induced culture medium of α-MP-T/pPICZα and α-MP/pPICZα transformants. Screening from 40 recombinant clones (grew on 1-2mg/ml Zeocin/YPD plates) revealed 6 clones which were found to express glucocerebrosidase. The specific activities on natural substrate ranged from 301.2 to 494.1nmol/hr/mg. No activity could be detected from the medium of the control transformants, or from the cell pellet fraction of those 6 recombinant clones that were found to secrete the recombinant glucocerebrosidase into the induced medium.

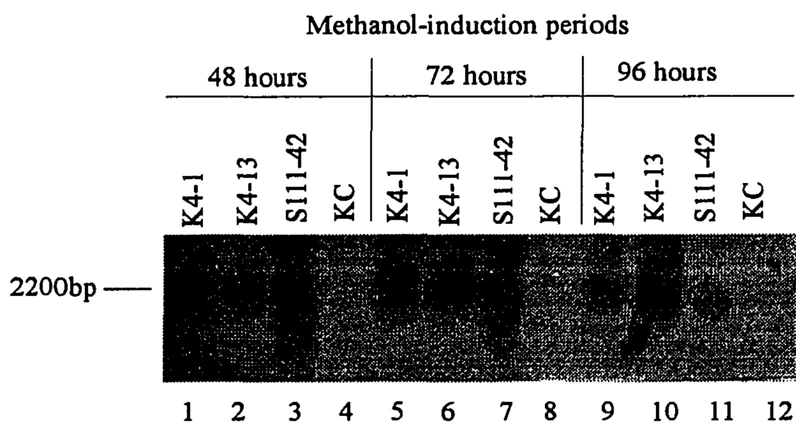


Figure 4.4 Northern blotting analysis of glucocerebrosidase gene in recombinant *Pichia pastoris*. KC is a recombinant control which transformed with vector only. K4-1, K4-13, and S111-42 are various recombinant yeast clones. Molecular size of RNA was determined using a RNA ladder from GIBCOL/BRL.

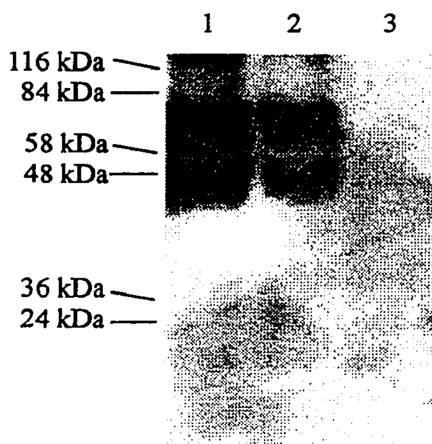


Figure 4.5 SDS-PAGE and Western-blotting of recombinant glucocerebrosidase from cell pellet fraction of induced recombinant clones that contain α-NTS-MP/pPIC9K construct (lane 1 and 2). Lane 3, a recombinant control, which contains vector only.

The Western blotting analysis of cell pellet fractions of recombinant clones that exhibited enzymatic activities demonstrated that the anti- glucocerebrosidase monoclonal antibody reacted to proteins of 110, 102, and 95 kDa (Figure 4.6). In contrast, the secreted form of recombinant glucocerebrosidase appeared to be 66 kDa (Figure 4.7).

4.4.3 Purification of recombinant glucocerebrosidase

The secreted recombinant glucocerebrosidase in the induced culture medium of clone K4-13 was purified to apparent homogeneity by hydrophobic chromatography, followed by anion-exchange chromatography. The recombinant enzyme was found to retain catalytic activity on 4MUGP substrate after purification. The purification was about 4.7-fold with respect to the culture medium. The yield was 7.2%. The results of a typical purification of recombinant glucocerebrosidase are shown in Table 4.1. Similar results were obtained in other separate experiments.

For the Phenyl column purification, although the column manufacturer recommended the use of 27% (w/v) or 2 M ammorium sulfate for adsorption of hydrophobic proteins to the column, we found that at such a high concentration, some of the recombinant enzyme would be precipitated or inactivated. After testing for different concentration preparations, we found when 0.85 M of ammonium sulfate was used, the enzyme activity level was not affected, and enzyme still adsorbed to the column.

During the hydrophobic chromatography, recombinant glucocerebrosidase was adsorbed by the Phenyl-Sepharose column during the enzyme application, and remained bound to the column during elution with the desalting gradient. Most recombinant glucocerebrosidase was eluted from the column when the elution solution was at 100% solution B (ddH_2O). Some of the enzyme remained bound to the column after ddH_2O

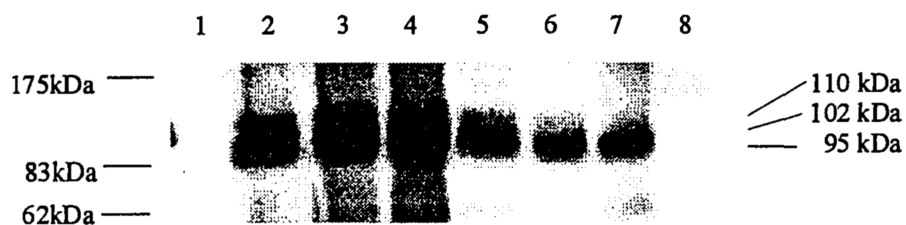


Figure 4.6 SDS-PAGE and Western-blotting of recombinant glucocerebrosidase from cell pellet fraction of induced recombinant clone K4-13. Lane 1 - 6, cell pellet fraction of K4-13, collected at different time points: 0, 24, 48, 72, 96, and 120 hours. Lane 7, K4-13 cell pellet fraction at 72 hours was treated with glycopeptidase F. Lane 8, cell pellet fraction of a control clone (host yeast only).

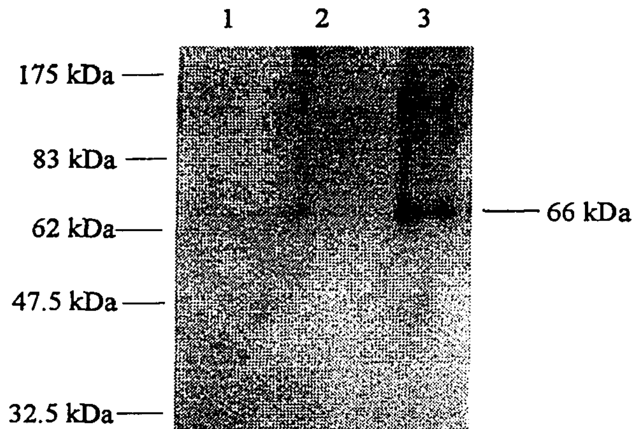


Figure 4.7 SDS-PAGE and Western-blotting of recombinant glucocerebrosidase from induction medium (BMMY). Lane 1, induction medium of a normal control; Lane 2, unconcentrated induction medium of recombinant clone K4-13, containing a-MP-T/pPICZa construct; Lane 3, 20X concentrated induction medium of recombinant clone K4-13. Molecular weight of protein markers are indicated on the left of the figure.

Purification	Specific activity (nmol/hr/mg)	Purification (fold)	Yield (%)
1. Culture medium	254	1	baseline*
2. Phenyl-Sepharose	928	3.7	19.5
3. Q-Sepharose	1187	4.7	7.2

*100% of the specific activity from the beginning of purification.

Table 4.1 Purification of recombinant glucocerebrosidase

elution, and was only eluted at a cholate concentration of 0.5% during cholate gradient elution (Figure 4.8). In the Q-Sepharose ion-exchange column chromatography, the enzymatic fractions were eluted out of the column when the elution solution reached to 100% solution B (20mM Bis-Tris and 1M NaCl, pH 7.0) (Figure 4.9).

To verify the molecular weight of purified recombinant glucocerebrosidase and purification efficiency, the final enzyme preparation was analyzed in a SDS-PAGE gel. Silver staining revealed the presence of one band at molecular weight of 66 kDa under denaturing conditions. This revealed that recombinant glucocerebrosidase of the expected size was purified to apparent homogeneity (Figure 4.10).

4.4.4 Biochemical and kinetic properties of recombinant glucocerebrosidase, and carbohydrate characterization

Substrate specificity (K_m) and pH profile. The partially purified recombinant glucocerebrosidase shows catalytic activity toward the synthetic substrate 4MUGP. The enzyme activity follows Michaelis-Menten kinetics with a K_m of 2.2 mM for 4MUGP (Figure 4.11), while the K_m of native enzyme from crude extracts of human fibroblast was 4 mM (Figure 4.12). The purified enzyme showed a similar pH optimium for hydrolysis of 4MUGP with that of native enzyme (Figure 4.13).

Effect of inhibitor. The partial purified recombinant glucocerebrosidase was found to be significantly inhibited by conduritol B-epoxide (CBE), a covalently bound inhibitor. After pre-incubation of the recombinant glucocerebrosidase with CBE at final concentration of 2.85 μM for 1hr at 4 ^{0}C, a complete loss of catalytic activity toward the natural substrate was observed; In contrast, the activity of control (untreated sample) was

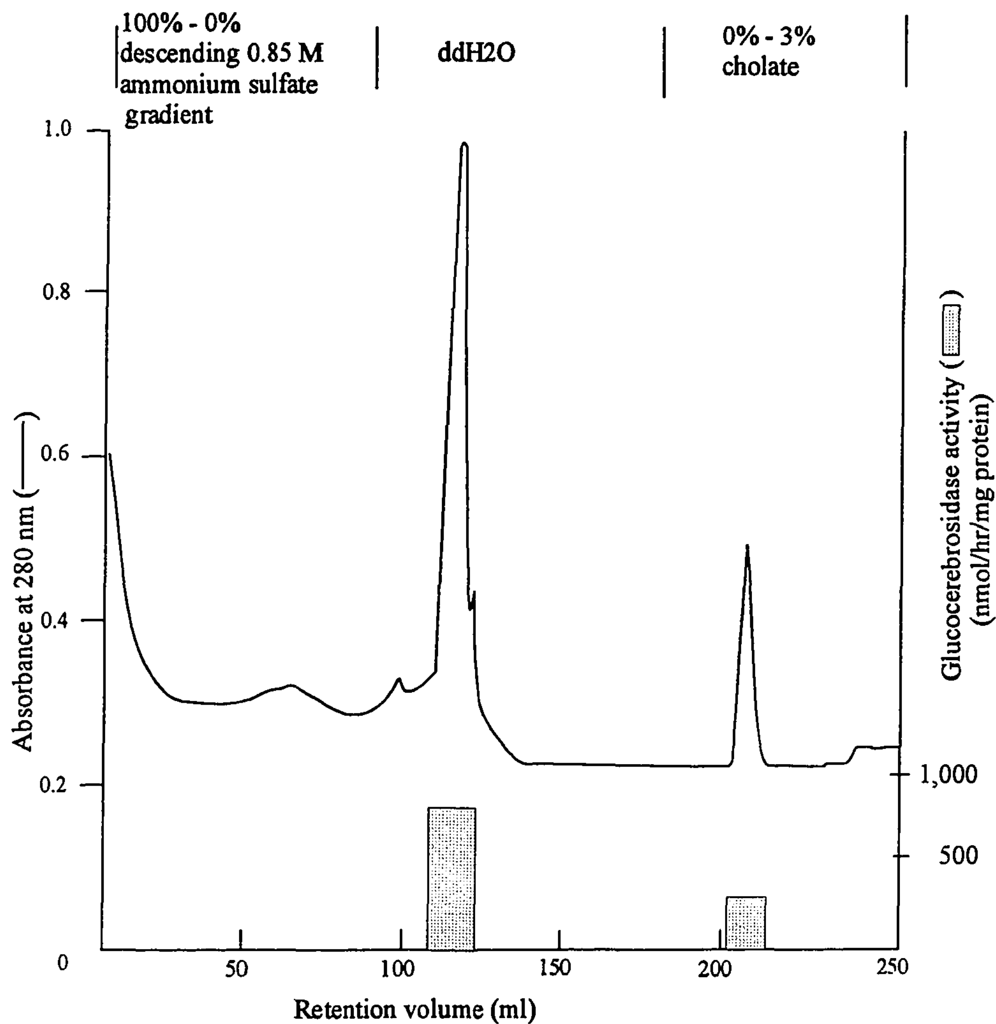


Figure 4.8 Phenyl-Sepharose hydrophobic interaction chromatography of recombinant glucocerebrosidase. The enzyme activity was determined using natural substrate N-palmitoyl-DL-dihydro-glucocerebroside.

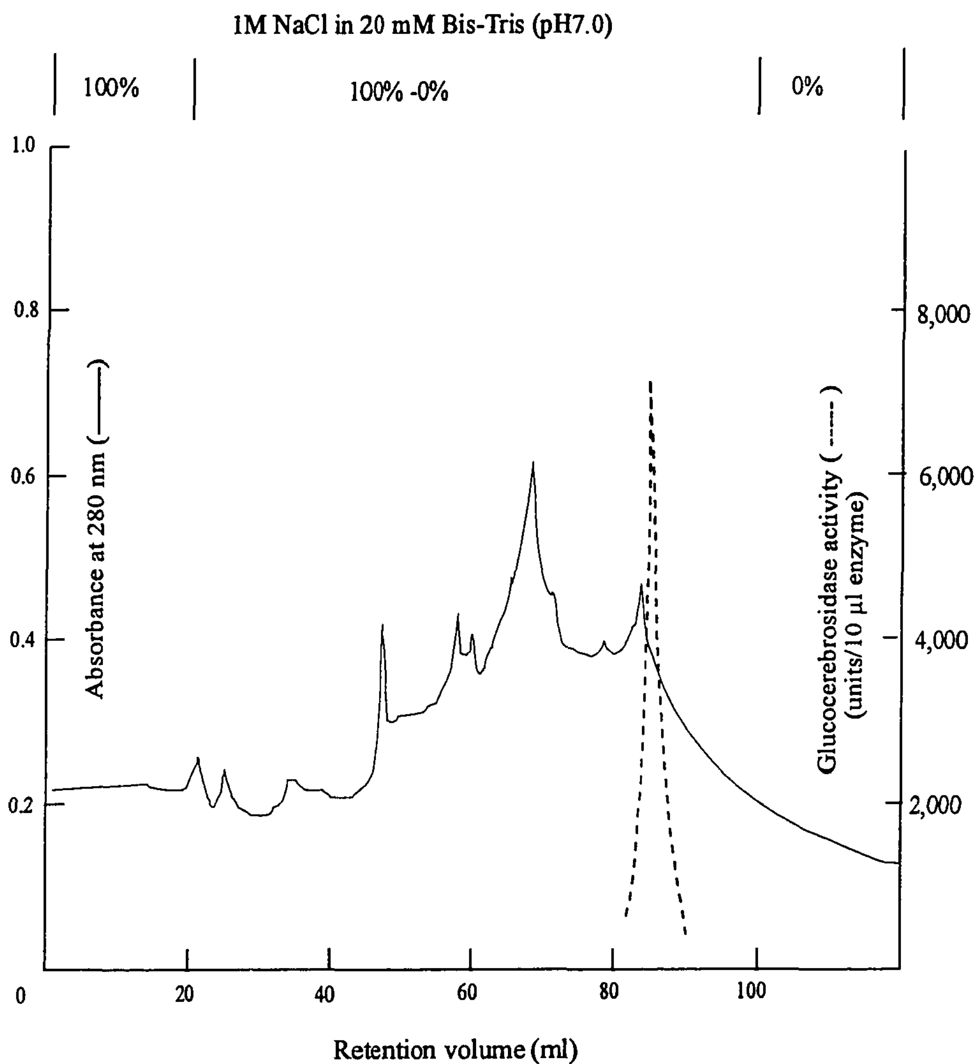


Figure 4.9 Q-Sepharose HR10/10 ion exchange chromatography of recombinant glucocerebrosidase. The enzyme activity was determined using 4MUGP as a substrate.

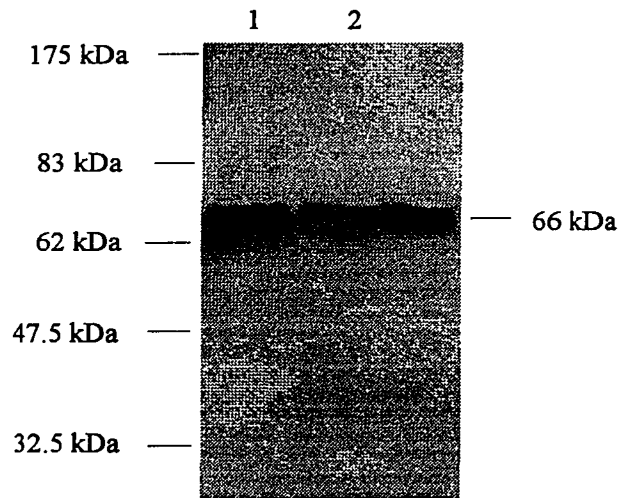


Figure 4.10 SDS-PAGE and silver-staining of purified recombinant glucocerebrosidase from Q-Sepharose ion-exchange chromatography (lane 1 and lane 2). Molecular weight of protein markers are indicated on the left of the figure.

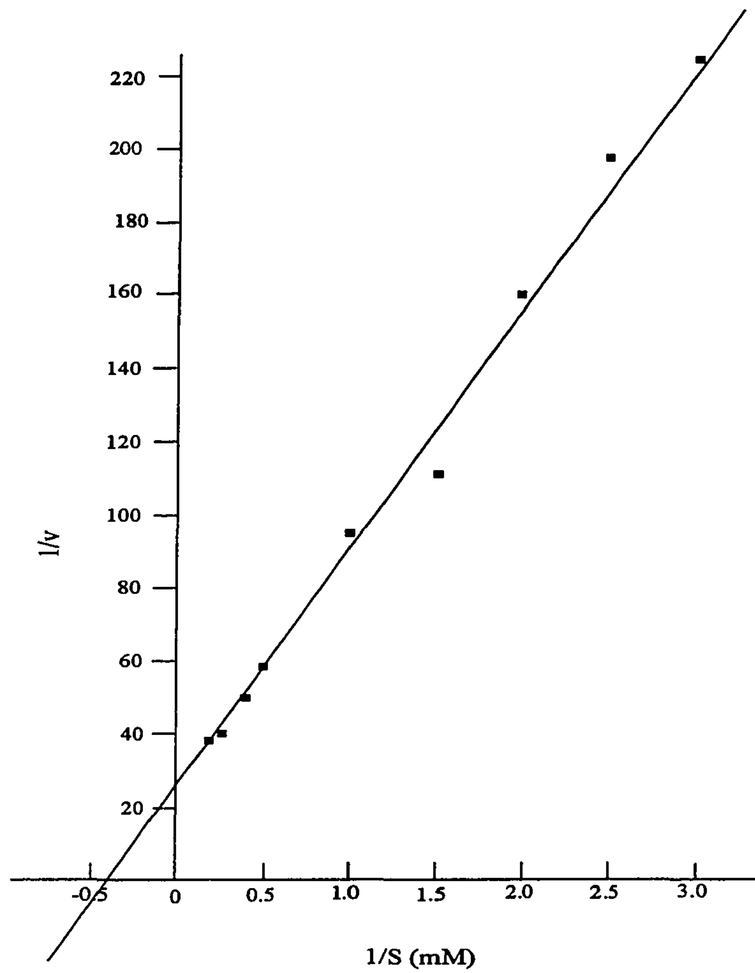


Figure 4.11 Lineweaver-Burk plot of glucocerebrosidase activity from recombinant enzyme. V is expressed as 4MU (millimoles) liberated/hr/mg protein.

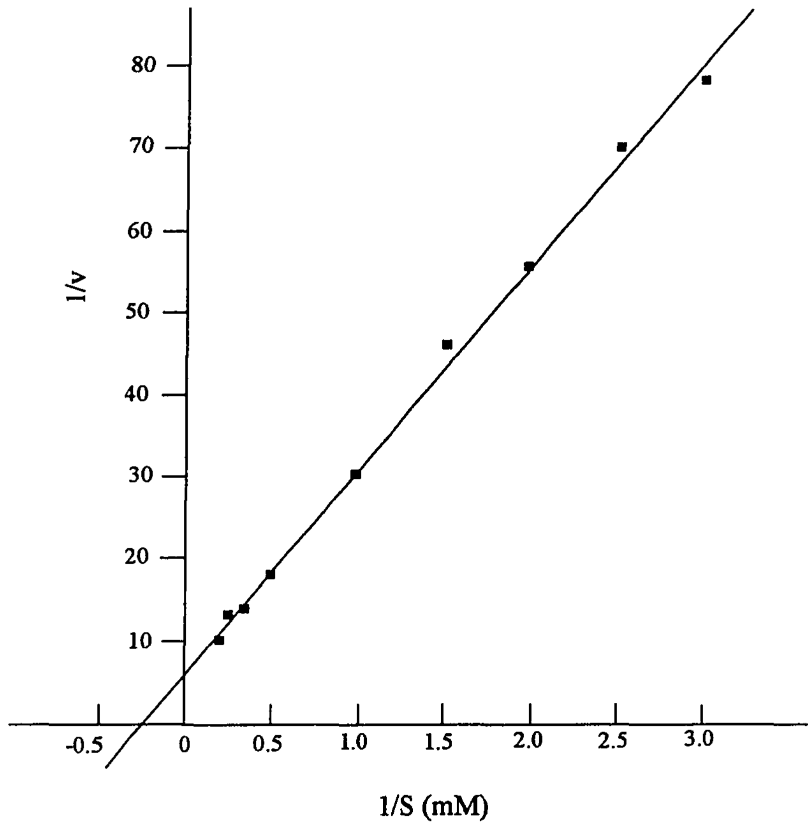


Figure 4.12 Lineweaver-Burk plot of glucocerebrosidase activity from human fibroblast. V is expressed as 4MU (micromoles) liberated/hr/mg protein.

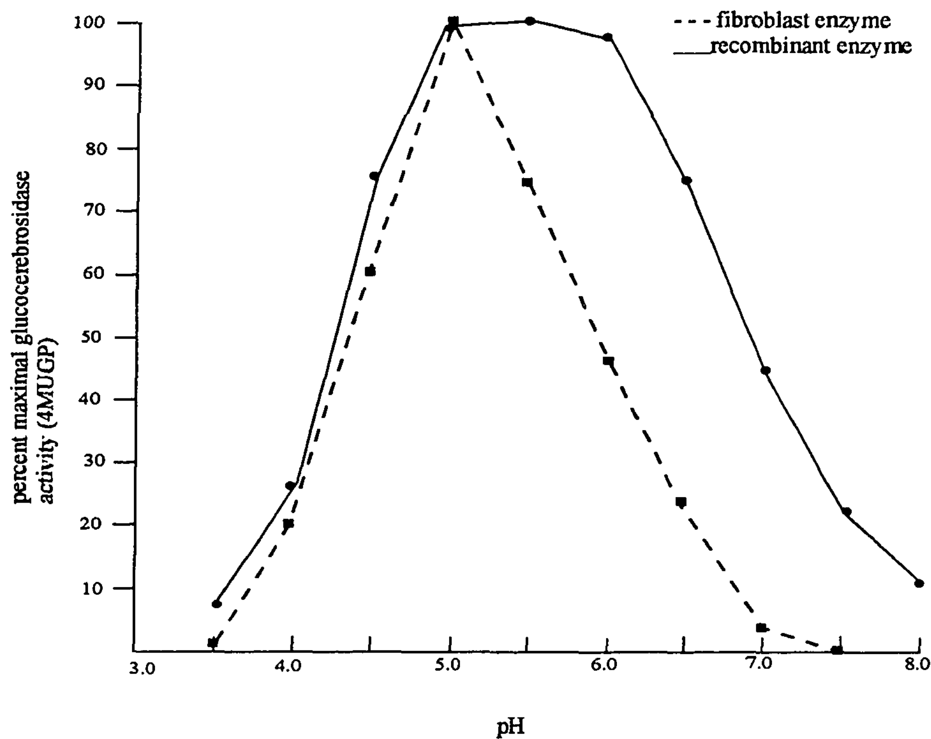


Figure 4.13 Comparison of pH profiles of human fibroblast glucocerebrosidase to recombinant enzyme. Assays were performed using 4MUGP as a substrate.

431.0 nmol/hr/mg. CBE also significantly inhibited recombinant glucocerebrosidase activity toward 4MUGP substrate. IC_{50} was found to be 0.4 μM (Figure 4.14).

Effect of detergent sodium taurocholate. The different concentrations of sodium taurocholate, ranging from 0.5% to 5% were tested for their effects on 4MUGP activity assay of recombinant glucocerebrosidase and the native enzyme from human fibroblast. It was observed that when the concentration of sodium taurocholate was between 0.5 to 2%, the enzymatic activities of both recombinant and native glucocerebrosidase reached their optimum.

Endoglycosidase deglycosylation. The partially purified secreted glucocerebrosidase, after treatment with peptide: N-glycopeptidase F, was applied to a 10% SDS-PAGE gel. It showed that N-linkage carbohydrate side chains were partial removed from the recombinant enzyme. The molecular weight of glucocerebrosidase after digestion with peptide N-glycopeptidase F was reduced to 58 kDa, which agreed with the expected size of deglycosylated recombinant glucocerebrosidase (Figure 4.15). However a unexpected band appeared at 42 kDa. It is speculated that this may be due to the presence of a contaminant protein, or protease contamination in the enzyme peptide: N-glycopeptidase F.

4.5 Discussion

Our study indicates that the construction of recombinant vectors is a critical step in the expression of glucocerebrosidase in *P. pastoris*. It has been shown that the glucocerebrosidase gene has two functional ATG initiator codons (Sorge *et al.*, 1987). The glucocerebrosidase cDNA insert within yeast transformants generated by α-NTS-MP/pPIC9K recombinant vector contains both of the ATG initiator codons. Although the

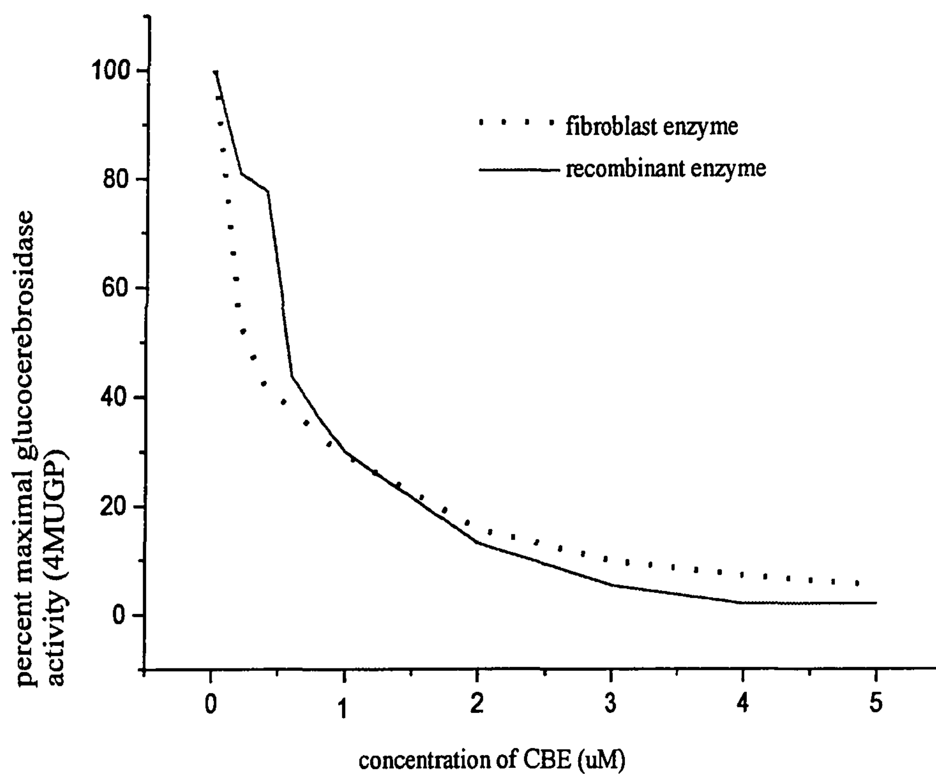


Figure 4.14 Effects of inhibitor conduritol B-epoxide on human fibroblast glucocerebrosidase and recombinant enzyme. Assays were performed using 4MUGP as a substrate.

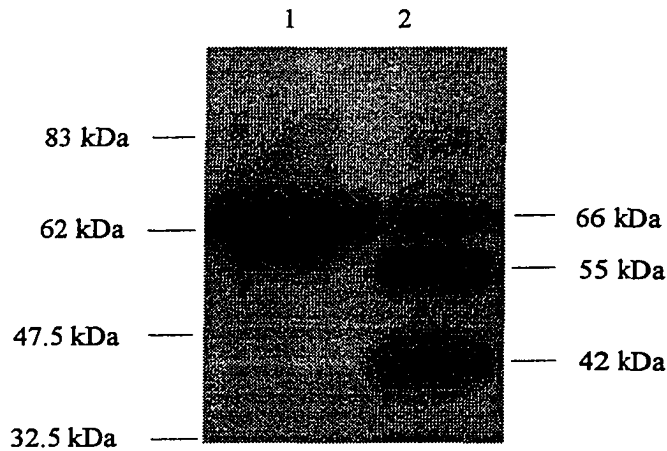


Figure 4.15 SDS-PAGE of deglycosylated recombinant glucocerebrosidase. Lane 1, control, untreated purified enzyme; Lane 2, purified enzyme treated with glycopeptidase F. An additional band with MW of 42 kDa, that was found in lane 2, may be resulted from protease comtamination in the enzyme reaction mixture. The molecular weight of protein markers are indicated on the left of the figure.

insert was cloned into the pPIC9K vector downstream from the ATG initiator codon of *AOX1* and α-secretion factor, there is a chance that translation may be initiated at one of native glucocerebrosidase ATG codons (Sorge *et al.*, 1987). Furthermore, if translation was initiated on the ATG initiator codon of *AOX1*, it would result in synthesis of a recombinant protein with two leader sequences, and would complicate targeting of the enzyme. This may explain why the recombinant enzyme was truncated in the cell pellet fraction and had no functional activity. When the native leader sequence was deleted, as in the constructs α-MP-T/pPICZα and α-MP/pPICZα, recombinant enzyme was expressed and secreted into the culture medium, although recombinant glucocerebrosidase was still detectable in cell pellet fractions by Western blotting analysis. It is speculated that the membrane associated recombinant enzyme may result from partial hyperglycosylation of the enzyme.

The culture conditions for the recombinant yeast clones were optimized using the shake flask culture. It has been demonstrated that optimization of growth parameters of the culture can be critical for the expressions of some heterologous proteins (Buckholz *et al.*, 1991; Cregg *et al.*, 1993). Previous reports also demonstrated that the addition of casamino acids and 5 mM EDTA enhance product stability (Clare *et al.*, 1991; Sreekrishna *et al.*, 1997). It was noted that under the shake flask culture condition, the pH in the inducing medium increased from 5 to 7.5 within 24 hrs of induction. The addition of 1M citrate buffer (pH 4.0) was needed to bring the pH level down to 5. When the pH level was maintained at this level (pH 5.0), a higher level of enzyme specific activity was detected. It has been reported that acidic conditions eliminate the problem of protein degradation by pH neutral active proteases (Clare *et al.*, 1991).

Glucocerebrosidase has been purified from placenta or fibroblasts in several different labs (Grabowski and Dagan, 1984; Choy, 1986; Aerts *et al.*, 1986; Osiecki-Newman *et al.*, 1986). The size of purified native enzyme is about 65 kDa (Choy, 1986; Barranger and Ginns, 1989; Beulter and Grabowski, 1995). The size of the partially purified recombinant enzyme from the *Pichia* expression system was identified as 66 kDa. It indicates that the protein expressed in our system is not hyperglycosylated. The structures of the carbohydrate side chains of the recombinant enzyme need to be analyzed to determine if there is any potential for antigeneicity.

Comparing the recombinant glucocerebrosidase, expressed in the *Pichia* expression system, with the native enzyme and recombinant enzyme expressed in the baculovirus expression system, the most significant difference is the solubility of the enzyme. The native enzyme and the baculovirus-expressed enzyme were found to be strongly membrane-bound (Choy and Davison, 1978; Grabowski *et al.*, 1989). The detergent taurocholate was required to solubilize the enzyme from cell membranes, while the *Pichia* expressed enzyme was soluble and secreted into the induced culture medium. However, the taurocholate assay revealed that in the presence of 0.5 - 2.5% of sodium taurocholate, the activity of *Pichia*- expressed enzyme increased. Choy (1990) reported that with the native enzyme adsorbed to hydrophobic resin, it was only eluted out of the column when the elution solution contained 0.6% sodium taurocholate (Choy and Woo, 1990). Most of the *Pichia* expressed enzyme that had adsorbed to the hydrophobic resin was eluted out of the column before sodium taurocholate was applied. It was presumed that the different properties of hydrophobic resins and the different solubility of enzymes might contribute to the different chromatography profiles during the enzyme purification. However, it was

noted that by increasing the ammonium sulfate concentration from 0.85 M to 1.7 M (Choy and Lin, unpublished observation), recombinant glucocerebrosidase was completely adsorbed during the desalting gradient elution. It exhibited the same binding property as the native placental enzyme and was eluted at 0.6% sodium taurocholate concentration. This modification also improved the yield from 7.2% to more than 60%.

The characterization studies of the recombinant enzyme indicated that recombinant glucocerebrosidase properties are equivalent to those of native glucocerebrosidase. The *Pichia* expressed enzyme had a K_m value of 2.2 mM when 4MUGP was used as a substrate, close to that reported for normal spleen glucocerebrosidase K_m = 8 mM (Basu *et al.*, 1984), normal placenta glucocerebrosidase (K_m = 1.6 mM) (Choy, 1980), and the baculovirus expressed enzyme (K_m =1.7 mM (Grabowski *et al.*, 1989). The optimum pH for the *Pichia* expressed enzyme is pH 5.0. This pH profile is similar to that of native enzyme, and to that of previous reports (Choy and Davison, 1980; Martin *et al.*, 1988). Comparing the effects of the specific active site inhibitor conduritol-B-epoxide on the *Pichia* expressed enzyme and native fibroblast enzyme, we found both were significantly inhibited, as was previously reported for the baculovirus expressed enzyme and other sources of native enzyme (Martin *et al.*, 1988).

From this study, it is demonstrated that a membrane-bound protein can be expressed in a secreted form into induced culture medium using the *Pichia* expression system. Although a wide variety of proteins have been expressed using the *Pichia* expression system, only a few are membrane-bound proteins, and were expressed intracellularly (Weiβ *et al.*, 1995; Talmont *et al.*, 1996). The α–factor secretion signal of *S. cerevisiae* has been used efficiently for the secretion of smaller-sized soluble proteins

such as mouse epidermal growth factor (Clare *et al.*, 1991) and single-chain antibody Fv fragment (Eldin *et al.*, 1977). Our results demonstrated that the α–factor secretion signal can also be utilized to direct a hydrophobic protein to be secreted into the culture medium. Another interesting finding is that that the epitope tag for protein detection and the histidine tag for the purification in the C-terminus of recombinant enzyme do not affect the functional characteristics of the expressed glucocerebrosidase. It should be noted that, in the case of expression of human tumor necrosis factor, a C-terminal tag with eight amino acid residues significantly affected the solubility of the expressed protein (Sreekrishna *et al.*, 1988).

There are reports indicating that multiple copies of the expression cassette are essential for high level expression. In other cases a single copy of the expression cassette is sufficient for optimal production (Cregg *et al.*, 1985; 1987). Thus, the effect of gene copy number on expression is unpredictable (Sreekrishna *et al.*, 1997). Since glucocerebrosidase is a membrane-bound protein, we predicted that it might require high copy number integration for optimal expression. An attempt to utilize the Zeocin resistance gene as a selection marker for screening for high copy number integration did not yield a satisfactory result. Colony numbers on each Zeocin plate decreased when the concentration of Zeocin increased from 0.25 to 2 mg/ml; but there was no difference in colony numbers when the concentration of Zeocin increased from 2 to 4 mg/ml. The result may indicate that this approach is not effective to differentiate the copy number of integration when certain copy numbers have been reached. Another concern is that Zeocin is a strong mutagen (Sreekrishna *et al.*, 1997). The potential effects of Zeocin on the DNA of host cells and DNA of integrants need to be considered. An assay on the relationship of copy number of

integration and expression level needs to be done for this particular enzyme. If it is necessary, the G418 resistance gene can be cloned into a pPICZα vector as a multiple copy selection marker (Scorer *et al.*, 1994). In G418 selection system, G418 resistance level of clones correlated to the number of multicopy integrants (Scorer *et al.*, 1994). Increased concentration of G418 in growth plates resulted in fewer, but higher multicopy number transformants. According to Scorer *et al* (1994), transformants that grow 4 mg/ml G418 contained 7 - 12 copies of integration cassette, whereas transformants containing one copy of integration cassette could only grow on 0.25 or 0.5 mg/ml G418.

In conclusion, this is the first description of the expression of glucocerebrosidase using *P. pastoris*. The results of this research indicate that *P. pastoris* is a suitable host for the functional expression of recombinant glucocerebrosidase. Further improvement to explore the full potential of this system may be achieved by screening multiple integrants in peptidase deficient strain SMD1163 and fermentation (Cregg *et al.*, 1993; Sreekrishna *et al.*, 1997), as well as manipulation of glycosylation pathway of yeast cells.

Chapter 5 Summary and Conclusion

Glucocerebrosidase is a lysosomal membrane-bound enzyme that hydrolyses the β-glucosidic linkage of glucocerebroside (Brady, 1965; Patrick, 1965). If the enzyme activity of glucocerebrosidase is profoundly deficient, the substrate will not be degraded to glucose and ceramide properly. As a consequence, it accumulates in the lysosomes of cells, and causes the disorder known as Gaucher disease. Gaucher disease is the most prevalent lysosomal storage disease. It is transmitted as an autosomal recessive trait (Barranger and Ginns, 1989). Based on the clinical severity, this disease was divided into three clinical subtypes: type 1, nonneuronopathic; type 2, acute neuronopathic; type 3, subacute neuronopathic (Knudson and Kaplan, 1962). The principle difference between those subtypes is the presence and progression of the neurological complications (Glew *et al.*, 1988; Barranger and Ginns, 1989).

The gene for glucocerebrosidase is located on chromosome 1q21 (Devine *et al.*, 1982; Ginns *et al.*, 1985). The sequences of the entire functional gene and its pseudogene have been determined (Horowitz *et al.*, 1989), and the cDNA was also cloned and sequenced (Sorge *et al.*, 1985; Tsuji *et al.*, 1986). Mutations in the gene have been identified and have been shown to result in proteins with deficient enzyme activities (reviewed by Beutler and Gelbart, 1998). It has been estimated, however, that nearly 25% of mutant alleles remain unidentified (Beutler *et al.*, 1990, 1992; Horowitz *et al.*, 1993). Enzyme replacement therapy has been known to be effective for treating type 1 Gaucher disease. However, the cost of treatment is prohibitively expensive. It is desirable to have an alternative approach for large–scale production of therapeutic recombinant glucocerebrosidase. The objectives of this thesis were 1) to identify unknown mutations

among various mutant Gaucher alleles; 2) to demonstrate if novel mutations were causative for enzyme deficiency; 3) to evaluate the feasibility of heterologous expression of glucocerebrosidase using the *Pichia* expression system.

DNA sequence analysis of twelve mutant alleles among six Gaucher patients revealed two novel mutations (649T and 1366G). The mutation 649T, from a type 2 Gaucher patient, resulted in Pro to Ser substitution at amino acid residue 178. Mutation 1366G, from a type 1 Gaucher patient, resulted in Phe to Val substitution at amino acid residue 417. Two rare mutations (481T and 1604A) were also identified from two type 1 Guacher patients. These two novel mutations, as well as the two rare mutations, either created or abolished endonuclease digestion sites. By utilizing the PCR amplification and RFLP analysis, we were able to develop a simple method for the identification of those mutations.

To characterize of mutant glucocerebrosidase alleles, the full-length of glucocerebrosidase cDNA was cloned into plasmid vector pAcUW1 at the *Bgl*II site downstream from the *AcMNPV p10* promoter. Both recombinant vector and baculovirus *AcUW1.lacZ* DNA were co-transfected into *Sf9* cells using the calcium phosphate-mediated transfection method. After plaque purification and virus amplification, the recombinant baculovirus was used to infect fresh *Sf9* cells. The activities of recombinant glucocerebrosidase from cell homogenates were measured using the fluorogenic substrate 4MUGP.

The enzyme activity from the positive control was 352.0 nmol/hr/mg protein, which was expressed from a normal allele. No endogenous glucocerebrosidase activity was detected in culture cells transfected by wild type baculovirus. Two well-characterized

alleles, 1226G and 1448C, were also cloned and expressed as references. The activity for 1226G was 23.1% of normal, and that for 1448C was 3.3%. This result was similar to previously published results and indicated the reliability of our expression system. The enzyme activities from the transfected *Sf9* cells with the Gaucher alleles (649T, 1366G, 481T, and 1604A) ranged from 2.8% to 17.3% of that expressed by the normal alleles.

To explore the feasibility of the heterologous expression of glucocerebrosidase in the yeast *Pichia pastoris*, the glucocerebrosidase cDNA without its native targeting signal was cloned into vector pPICZα, and the recombinant cDNA/vector construct was transformed into yeast *P. pastoris* KM71 strain. Under an induction condition, the recombinant glucocerebrosidase was expressed and secreted into the culture medium. The maximum expression level under flask culture conditions reached a specific activity of 494 nmol/hr/mg protein. The presence of recombinant glucocerebrosidase was also detected by Western blot analysis. By using a successive two-step FPLC procedure (Phenyl Sepharose hydrophobic interaction and anion exchange chromatographies), the recombinant enzyme was purified to apparent homogeneity. The size of the secreted form of recombinant glucocerebrosidase was determined to be 66 KDa.

Based on the data from Lineweaver-Burk plots, K_m value for recombinant enzyme was 2.2 mM, while that of native enzyme from human fibroblast was 4.0 mM. These data are comparable with previous publications (Choy *et al.*, 1980; Grabowski *et al.*, 1989; Cormand *et al.*, 1997). The V_{max} of recombinant enzyme was 40,000 nmol/hr/mg, while that of crude extracts of fibroblast was 167 nmol/hr/mg. The IC_{50} of the irreversible inhibitor, conduritol-B-epoxide, was 0.35 μM for the native enzyme and 0.55 μM for the

recombinant enzyme. The purified recombinant enzyme showed a pH profile for hydrolysis of 4MUGP similar to that of the native enzyme.

The results of the study on mutation identification revealed two novel mutations (1604A and 1366G). The characterization study on mutant Gaucher alleles demonstrated that the two novel mutations (1604A and 1366G) and the two rare mutations (481C and 1604A) are deleterious and resulted in profoundly deficient in glucocerebrosidase activities. The result of the heterologous expression of glucocerebrosidase using the *Pichia* expression system indicated that *P. pastoris* can be used for the expression of the recombinant glucocerebrosidase, and the kinetic properties of the recombinant enzyme were similar to that of the native enzyme.

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