STABILIZATION AND MECHANISTIC STUDIES OF SOLUBLE PROCESSING ALPHA-GLUCOSIDASE I FROM *SACCHAROMYCES CEREVISIAE*

by

PATRICK YING XIA LI

B.Sc.(Honours), The Memorial University of Newfoundland, 1996

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Food Science

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1999

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Department of Food Science

The University of British Columbia
Vancouver, Canada

Date Oct. 14, 1999

Abstract

Processing α-glucosidase I (E.C.3.2.1.106) is a key enzyme in the biosynthesis of asparagine-linked oligosaccharides catalyzing the first processing event after the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ to proteins. The stability and mechanism of this enzyme were investigated in this research. The enzyme was isolated from dry yeast cells and was further purified using affinity chromatography with an enzyme inhibitor, N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, as the ligand, and Concanavalin A-Sepharose chromatography. To improve the long-term stability of the enzyme, various additives were added into the phosphate buffer used for extraction and isolation. These additives were different protease inhibitors, including protease inhibitor cocktail [Sigma, product No. P8215, containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane(E-64), and 1,10-phenanthroline] (1%) and phenylmethylsulfonyl fluoride (PMSF) (100 μM); reducing agent - dithiothreitol (DTT) (0.5 mM); chelating agent - ethylenediaminetetraacetic acid (EDTA) (0.2 mg/ml); polyol - glycerol (10%); and protective protein solution - bovine serum albumin (BSA) (1 mg/ml). The stability of the isolated enzyme was studied when kept at 4°C, -25°C, -80°C, and after freeze drying. Kinetic evaluation of several synthesized substrate analogues and chemical modification of the active site of the enzyme were also attempted. It was showed in this research that processing α-glucosidase I could be further purified using Concanavalin A-Sepharose chromatography. The stability of the enzyme could be much improved by the addition of glycerol, EDTA, BSA, PMSF and protease inhibitors during isolation. Extremely low freezing temperature (-25°C and -80°C) could help to retain enzyme activity during storage. Processing α-glucosidase I was an inverting enzyme which catalyzes

hydrolysis with inversion of anomeric configuration. Synthetic substrate analogues 2'Fluoro-αGlc(1-2)αGlc-O-grease and 2'N$_3$-αGlc(1-2)αGlc-O-grease were competitive inhibitors of the enzyme and at least one acidic amino acid might be present at the active site of the enzyme. It is believed that results from this research will provide useful information in the design of appropriate enzyme inhibitors that could be beneficial to human health.

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1. Introduction

Asparagine-linked (or N-linked) glycoproteins are a group of biologically important proteins. In animal cells, most membrane receptors, including those for low density lipoproteins (LDL), for insulin, and for acetylcholine, are asparagine-linked glycoproteins, as are many other biologically important proteins such as lysosomal enzymes, serum proteins and secreted proteins (Elbein, 1991a).

The carbohydrate portions of these proteins are assembled via the dolichol cycle in the rough endoplasmic reticulum (ER) (Kornfeld and Kornfeld, 1985). There are three major classes of N-linked oligosaccharides - the high-mannose, the complex, and the hybrid types (Fig.1), which share a common biosynthetic pathway, and which contain a common core region of:

$$\text{Man}\alpha 1,6(\text{Man}\alpha 1,3)\text{Man}\beta 1,4\text{-GlcNAc}\beta 1,4\text{-GlcNAc}$$
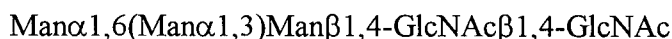
All of the various N-linked oligosaccharides arise from the common intermediate, $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol, a lipid-linked oligosaccharide that is assembled in the ER of the cell by the sequential addition of the sugars N-acetyl-D-glucosamine, mannose, and glucose to the lipid carrier, dolichol-P (P=pyrophosphate) (Hubbard and Ivatt, 1981). The oligosaccharide is then transferred from this lipid carrier to specific asparagine residues that are in the consensus sequence, Asn-X-Ser(Thr). Once the oligosaccharide is transferred to protein, the oligosaccharide chain begins to undergo a series of processing events (Fig. 2). These events start in the rough ER with the trimming of the glucosyl residues and continue in the Golgi compartment where one or more mannosyl residues are also clipped. Depending on individual glycoproteins, additional glycosylations of the trimmed oligosaccharide, mediated by the Golgi-localized glycosyltransferases, may

1

(a)

Manα1→2Manα1 ↘6
              Manα1 ↘6
Manα1→2Manα1 ↗3   Manβ1→4GlcNAcβ1→4GlcNAc→Asn
                      ↗3
Manα1→2Manα1 → 2 Manα1 ↗

(b)

SA→Gal→GlcNAc→Manα1 ↘6
                      Manβ1→4GlcNAcβ1→4GlcNAc→Asn
                      ↗3
SA→Gal→GlcNAc→Manα1 ↗

(c)

Manα1 ↘6
        Manα1 ↘6
Manα1 ↗3   Manβ1→4GlcNAcβ1→4GlcNAc→Asn
            ↗3
SA→Gal→GlcNAc→Manα1 ↗
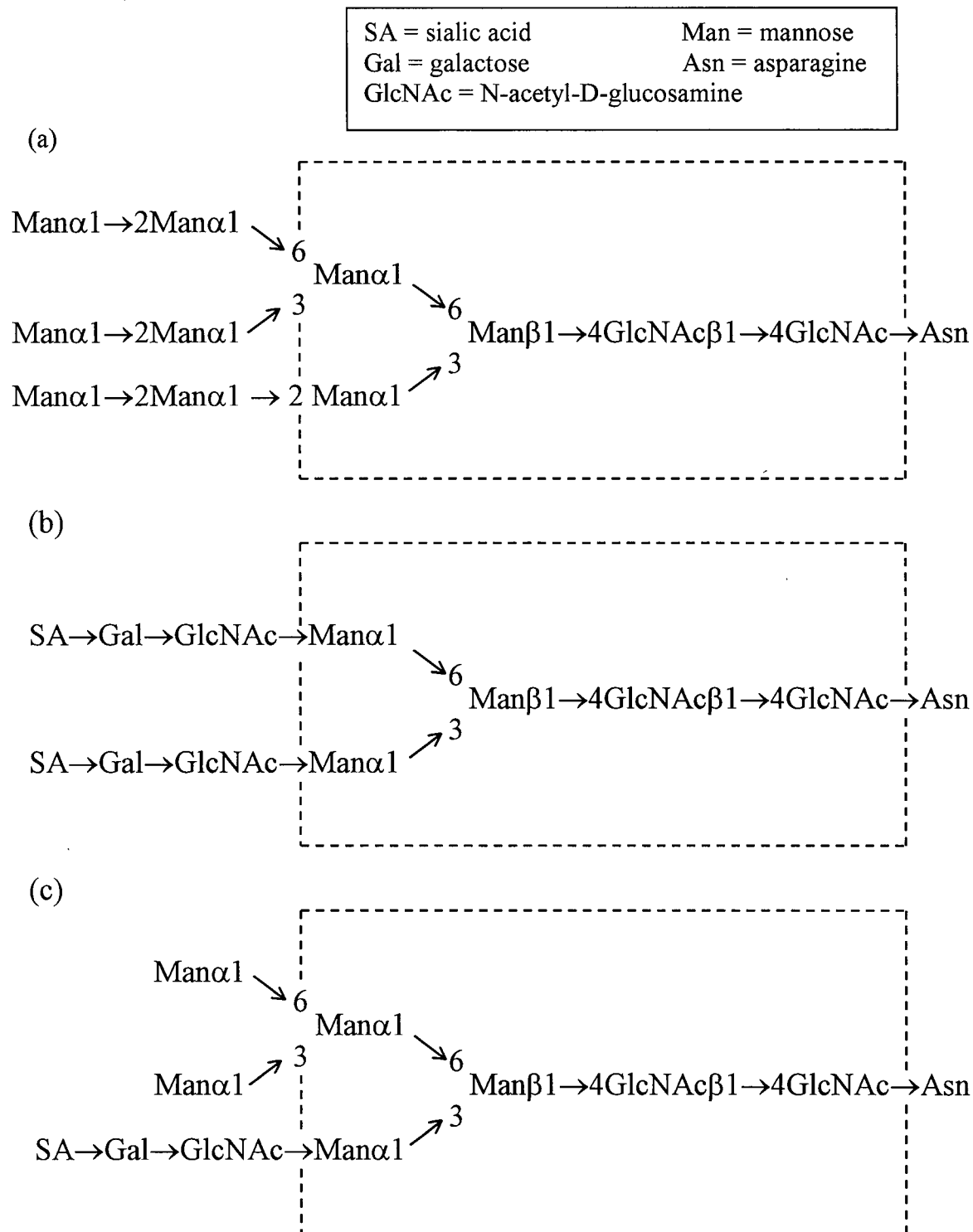
Figure 1. Structures of the typical N-linked oligosaccharides. (a) The high-mannose, (b) the complex and (c) the hybrid types of oligosaccharides. Boxed is the common core.

(Glc)₃(Man)₉(GlcNAc)₂-PP-Dol

$(Glc)_3(Man)_9(GlcNAc)_2$-PP-Dol

Man-Man
                Man
Man-Man   Man-GlcNAc-GlcNAc-Asn

Glc-Glc-Glc-Man-Man-Man

Glc ⟵—— ↓ *Glucosidase I*

Glc ⟵—— ↓ *Glucosidase II*

Man-Man
                Man
Man-Man   Man-GlcNAc-GlcNAc-Asn → High mannose
                                                                     chains
Man-Man-Man

Man ⟵—— ↓ *Mannosidase I*

Man
          Man
Man   Man-GlcNAc-GlcNAc-Asn

Man

GlcNAc ⟶ ↓ *GlcNAc Transferase I*

Man
Hybrid ⟵   Man
chains
Man   Man-GlcNAc-GlcNAc-Asn

GlcNAc-Man

Man ⟵—— ↓ *Mannosidase II*

Man
            Man-GlcNAc-GlcNAc-Asn
GlcNAc-Man

↓

↓

↓

SA-Gal-GlcNAc-Man
                        Man-GlcNAc-GlcNAc-Asn
SA-Gal-GlcNAc-Man

Figure 2. The processing pathway for modification of the N-linked oligosaccharides.

occur to yield the final oligosaccharide moieties in the proteins (Kornfeld and Kornfeld, 1985).

As the first processing enzyme, processing α-glucosidase I (glucosidase I) triggers the onset of the above processing events by removing the outermost α1,2-linked glucose from the newly constructed glycoprotein. It is therefore an important factor in the overall regulation of glycoprotein biosynthesis. Some researchers are examining its role in promoting protein targeting and folding (Faye *et al.*, 1993). Some researchers are studying its role in the biosynthesis of cell wall components (Abeijon and Chen, 1998; Shahinian *et al.*, 1998).

Some glucosidase I inhibitors have been shown to act as antiviral and antitumor agents (Elbein, 1991b; Fenouillet *et al.*, 1994; Fischer *et al.*, 1995; van den Broek *et al.*, 1996). Many viruses are enveloped viruses. In most cases, the virus contains one or more glycoproteins that make up the envelope structure, and these glycoproteins are frequently of the N-linked type (Elbein, 1991b). Therefore, inhibiting the enzyme glucosidase I could affect N-linked glycosylation or block modifications in the processing of the N-linked oligosaccharides, and thus have profound effects on viral growth and replication. As well, N-linked glycoproteins are the components of the tumor cell surface involved in the recognition and lysis. This suggests that N-linked oligosaccharides participate in recognition and/or lysis events.

In other words, glucosidase I is an important enzyme in glycoprotein biosynthesis. Inhibitors of this enzyme are potential antiviral and anti-cancer agents. Better understanding of the characteristics of this enzyme will therefore facilitate development of inhibitors which could be used in treatment of some main human diseases including Acquired

Immunodeficiency Syndrome (AIDS) and cancers.

Investigations of the active site components of glucosidase I have recently started through studies on the structure-function relationships of glucosidase I (Pukazhenthi *et al.,* 1993; Romaniouk and Vijay, 1997). It is believed that this valuable information will provide important direction in the design of appropriate enzyme inhibitors that could be beneficial to human health (Palcic *et al.,* 1999).

The objectives of this research project were to improve techniques used to isolate and stabilize glucosidase I; to increase understanding of glucosidase I mechanism by revealing the stereochemistry of glucosidase I reaction, by evaluating the kinetic effect of substrate analogues, and by investigating the active site components of glucosidase I.

2. Literature Review

2.1. Purification of Glucosidase I

2.1.1. Isolation of Glucosidase I

2.1.1.1. Category of Enzymes and General Procedure of Isolating an Enzyme

Enzymes are an important group of proteins. They control the chemical reactions of life through their catalytic action. In general, enzymes can be classified into six categories: 1) oxidoreductases which are involved in biological oxidations and reductions; 2) transferases which catalyze the transfer of a group from one substrate to another; 3) hydrolases that catalyze the formation of two products from a substrate by a hydrolytic reaction; 4) lyases that catalyze the addition of groups to double bonds or their formation; 5) isomerases that catalyze isomerisation; and 6) ligases (or synthetases) which join together two molecules, simultaneously with the breakdown of ATP (Wiseman and Gould, 1971). Hydrolases are further classified into esterases, thiolesterases, phosphatases, glycosidases, and peptidases (Dixon and Webb, 1964). Glycosidases catalyze the hydrolysis of glycosidic bonds. Processing α-glucosidase I, the enzyme studied in this research work, is within this category. Glycosidases play a central role in a number of biological processes that are of significant interest for biochemistry, medicine, and biotechnology. For example, lysozyme is present in egg white and human tears and cleaves bacterial cell wall polysaccharides, thereby serving as a relatively broad range bactericidal agent (Withers and Aebersold, 1995); also, specific enzymes are increasingly used for food processing (invertase for production of "invert" sugar, cellulases for fruit juice processing, β-galactosidase for lactose reduction) (Coughlan and Hazlewood, 1993).

Enzymes are found in nature in complex mixtures, usually in cells that contain a

hundred or more different enzymes, and in order to study an enzyme properly it must be isolated. The serious isolation of enzymes can be dated back to 1920s (Dixon and Webb, 1964). The first crystalline enzyme (urease) was obtained by Sumner (1926). By 1940 about twenty highly purified enzymes had been obtained, and this process has continued at an ever-increasing rate. In the isolation of an enzyme, the general procedure usually includes: 1) determining a convenient quantitative test of activity by which the enzyme can be estimated. Since a large part of the time spent in enzyme isolation is used up in testing the activity of the different fractions, it is most desirable that the test method should be a rapid one; 2) searching for a rich source of the enzyme. The amount of an enzyme may be very different in different sources and by choosing a source which is rich in the enzyme not only is more enzyme obtained, but the degree of purification required to obtain the pure enzyme is much less, perhaps by a factor of 10 or even 100; 3) making an extract containing the enzyme from the chosen raw material. There are many methods to disintegrate cells because there are many different types of cell. Most cells have particular characteristics that need special attention during disintegration. Animal tissues vary from the very easily broken erythrocytes to tough collagenous material such as blood vessels and other smooth-muscle-containing tissue. Plant cells are generally more difficult to disrupt than animal cells because of the cellulosic cell walls. Bacteria vary from fairly fragile organisms that can be broken up by digestive enzymes or osmotic shock to more resilient species with thick cell walls, needing vigorous mechanical treatment for disintegration. Table 1 gives a list of techniques that can be used. These techniques have been illustrated in great detail (Scopes, 1982); 4) separating the enzyme from numerous other substances. The enzyme is usually separated by precipitation first. This can be done by change of pH, by heating, by salts, by selective

Table 1. Cell disintegration techniques (Adapted from Scopes, 1982).

Technique	Example	Principle
Gentle		
Cell lysis	Erythrocytes	Osmotic disruption of cell membrane
Enzyme digestion	Lysozyme treatment of bacteria	Cell wall digested, leading to osmotic disruption of cell membrane
Chemical solubilization / autolysis	Toluene extraction of yeast	Cell wall (membrane) partially solubilized chemically; lytic enzymes that are released complete the process
Hand homogenizer	Liver tissue	Cells forced through narrow gap, rips off cell membrane
Mincing (grinding)	Muscle etc.	Cells disrupted during mincing process by shear force
Moderate		
Blade homogenizer (Waring-type)	Muscle tissue, most animal tissues, plant tissues	Chopping action breaks up large cells, shears apart smaller ones
Grinding with abrasive (e.g., sand, alumina)	Plant tissues, bacteria	Microroughness rips off cell walls
Vigorous		
French press	Bacteria, plant cells	Cells forced through small orifice at very high pressure; shear forces disrupt cells
Ultrasonication	Cell suspensions	Micro-scale high-pressure sound waves cause disruption by shear forces and cavitation
Bead mill	Cell suspensions	Rapid vibration with glass beads rips cell walls off
Manton-Gaulin homogenizer	Cell suspensions	As for French press above, but on a larger scale

denaturation, with organic solvents, or with organic polymers. Then the enzyme can be further purified by adsorption and other more advanced techniques such as chromatography, gel filtration, electrophoresis, isoelectric focusing, liquid phase partitioning, and ultrafiltration, etc. Among these techniques, affinity chromatography is by far the most useful and successfully applied method for providing high purity proteins. For example, non-affinity methods generally provide less than a 20-fold purification of proteins, but affinity chromatography routinely achieves purification of between 1,000- and 10,000-fold. The basic principle of affinity chromatography is described in Fig.3.

2.1.1.2. Glucosidases and the Isolation of Glucosidase I

Glucosidases (E.C.3.2.1) belong to the category of hydrolases. Initial investigations on the processing of newly synthesized glycoproteins indicated the removal of glucose residues prior to mannose from lipid-linked oligosaccharides (Ugalde et al., 1980). Two glucosidase activities were subsequently identified (Chen and Lennarz, 1978; Grinna and Robbins, 1979; Elting et al., 1980) and termed glucosidase I (E.C.3.2.1.106), which removes the terminal $\alpha1,2$-linked glucose from $Glc_3Man_9GlcNAc_2$, and glucosidase II, which removes the remaining two $\alpha1,3$-linked glucose residues from the precursor.

As an important enzyme in the glycoprotein biosynthesis, glucosidase I has gained increasing attention in research. It has been isolated from various sources, including rat liver (Grinna & Robbins, 1979; Ugalde et al., 1980), mung bean seedlings (Szumilo et al., 1986), mammary tissue (Shailubhai et al., 1987; Shailubhai et al., 1991), pig liver (Bause et al., 1989), yeast (Bause et al., 1986; Neverova et al., 1994) and human hippocampus (Kalz-Fuller et al, 1995). Glucosidase I has been obtained in two forms. One form is membrane-bound and its activity was stimulated by detergent, whereas the other form was soluble

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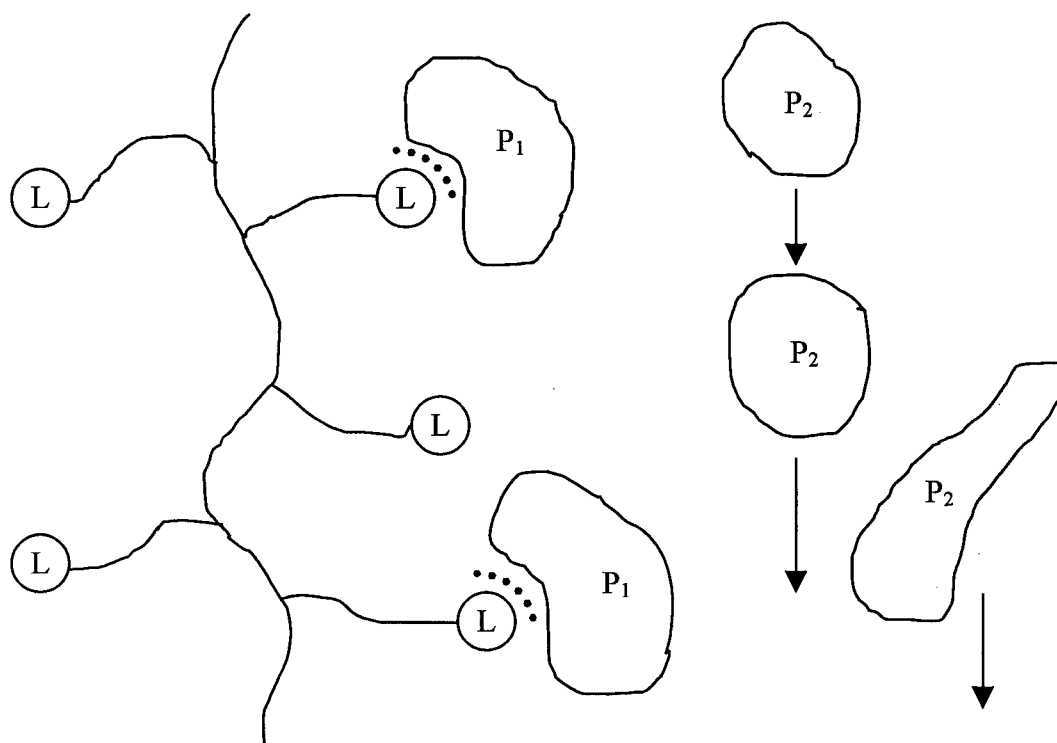
Figure 3. Basic principle of affinity chromatography. A ligand L is covalently attached to the backbone matrix. Only proteins P_1 with a specific affinity for L bind to the absorbent. Proteins P_2 pass through unaffected.

without detergent.

During the isolation steps of glucosidase I in these studies, the raw materials from different sources usually underwent a series of homogenization, filtration and centrifugation in extraction buffer. Then the microsomal glucosidase I (membrane-bound form) was solubilized with detergents such as Triton X-100 and Lubrol PX, or the soluble glucosidase I was precipitated out with ammonium sulfate. Both forms of crude enzyme were then subjected to chromatographic techniques. For example, in isolating microsomal glucosidase I from mung bean seedlings (Szumilo *et al.*, 1986), when the solubilized enzyme fraction was chromatographed on DE-52, glucosidase I activity was detected. The glucosidase I was purified about 200-fold by chromatography on hydroxylapatite, Sephadex G-200, and dextran-Sepharose. In isolating soluble glucosidase I from yeast cells (Neverova *et al.*, 1994), after the yeast cells were broken up by passing through a French press twice and cell debris were removed by centrifugation, the enzyme was isolated from a 20-60% ammonium sulfate protein fraction of the supernatant which was then applied to a Toyopearl DEAE column and a Mono-Q HR 5/5 column.

In addition to the isolation steps listed above, glucosidase I has also been highly purified in some studies using affinity adsorption chromatography. There are three types of affinity chromatography. These include the use of specific ligands (e.g., enzyme inhibitors, hormones, neurotransmitters), a general ligand (lectins), and immunoaffinity ligands (antibodies). With various derivatives of a potent glucosidase I inhibitor - 1-deoxynojirimycin as the specific ligand, including N-(5-carboxypentyl)-1-deoxynojirimycin (Hettkamp *et al.*, 1984; Bause *et al.*, 1986; Pukazhenthi *et al.*, 1993) and N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin (Bause *et al.* 1991) (refer to Fig.4 for structures),

(a)
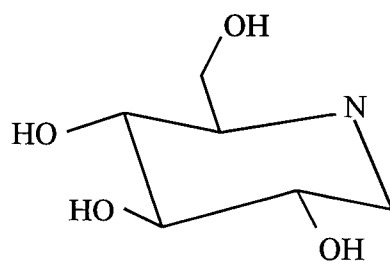
(b)
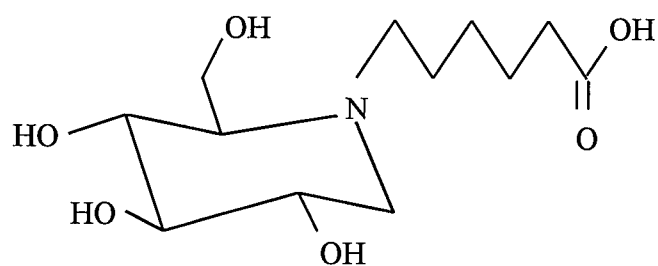
(c)
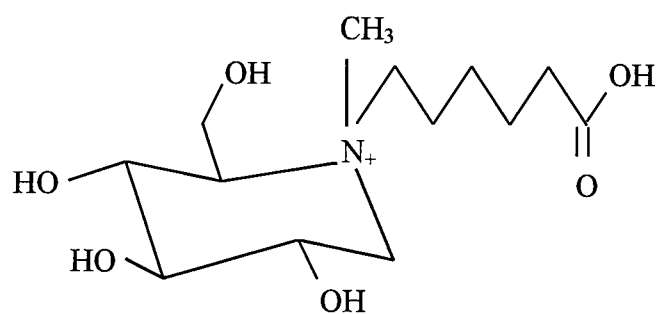
Figure 4. Structure of (a) 1-deoxynojirimycin, (b) N-(5-carboxypentyl)-1-deoxynojirimycin, and (c) N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin.

glucosidase I was purified from about 200-fold to about 960-fold over the crude

microsomal fraction. Since glucosidase I itself is a glycoprotein with high-mannose

oligosaccharide(s) (Bause *et al.,* 1986), Concanavalin A affinity chromatography has also

been used for further purification of glucosidase I (Bause *et al.,* 1986; Szumilo *et al.,* 1986)

and found to be able to purify the enzyme by about 200-fold to 1,900-fold depending on the

techniques used in the isolation steps. Concanavalin A is one type of lectin, which are

defined as carbohydrate-binding proteins of nonimmune origin, and it offers a rapid method

of purifying glycoproteins. At neutral pH concanavalin A exists as a tetramer with each

subunit possessing one specific carbohydrate-binding site (Sadhu and Magnuson, 1989). The

carbohydrate-binding site is specific for structures containing α-linked mannose or glucose.

Two pyranoses, α-D-mannopyranose and α-D-glucopyranose, are recognized for binding

by Concanavalin A, and the affinity of Concanavalin A toward α-D-mannopyranose is ca.

3.5-fold higher than toward α-D-glucopyranose (Hassing and Goldstein, 1970).

Two main methods have been used to assay the glucosidase I activity in those

studies. Initially, glucosidase I activity was monitored by incubating the enzyme preparation

with the natural substrate - $Glc_3Man_9GlcNAc_2$ obtained from microsomes or cell culture.

The Glc was randomly labeled using ^3H or ^{14}C. The release of $[^3H]$glucose or $[^{14}C]$glucose

was determined and one unit of enzyme activity was defined as the amount of enzyme

capable of releasing 1% $[^3H]$glucose or $[^{14}C]$glucose from the labeled oligosaccharide per

minute. In a recent study (Neverova *et al.,* 1994) a trisaccharide substrate, α-D-Glc1$\rightarrow$2α-

D-Glc1$\rightarrow$3α-D-Glc-O(CH$_2$)$_8$COOCH$_3$, was used to determine enzyme activity. The

terminal glucose was released from the substrate by the enzyme and quantitated using

glucose oxidase, peroxidase, and *o*-dianisidine (Fig.5). One unit of enzyme activity is

αGlc(1→2)αGlc(1→3)αGlc-O-R

Glucosidase-I

Reducing Glc   + αGlc(1→3) αGlc-O-R

2(H$_2$O),O$_2$ | *Glucose Oxidase*

Gluconic acid + H$_2$O$_2$

o-Dianisidine | *Peroxidase*
(colorless)

Oxidized o-dianisidine   (Brown color)
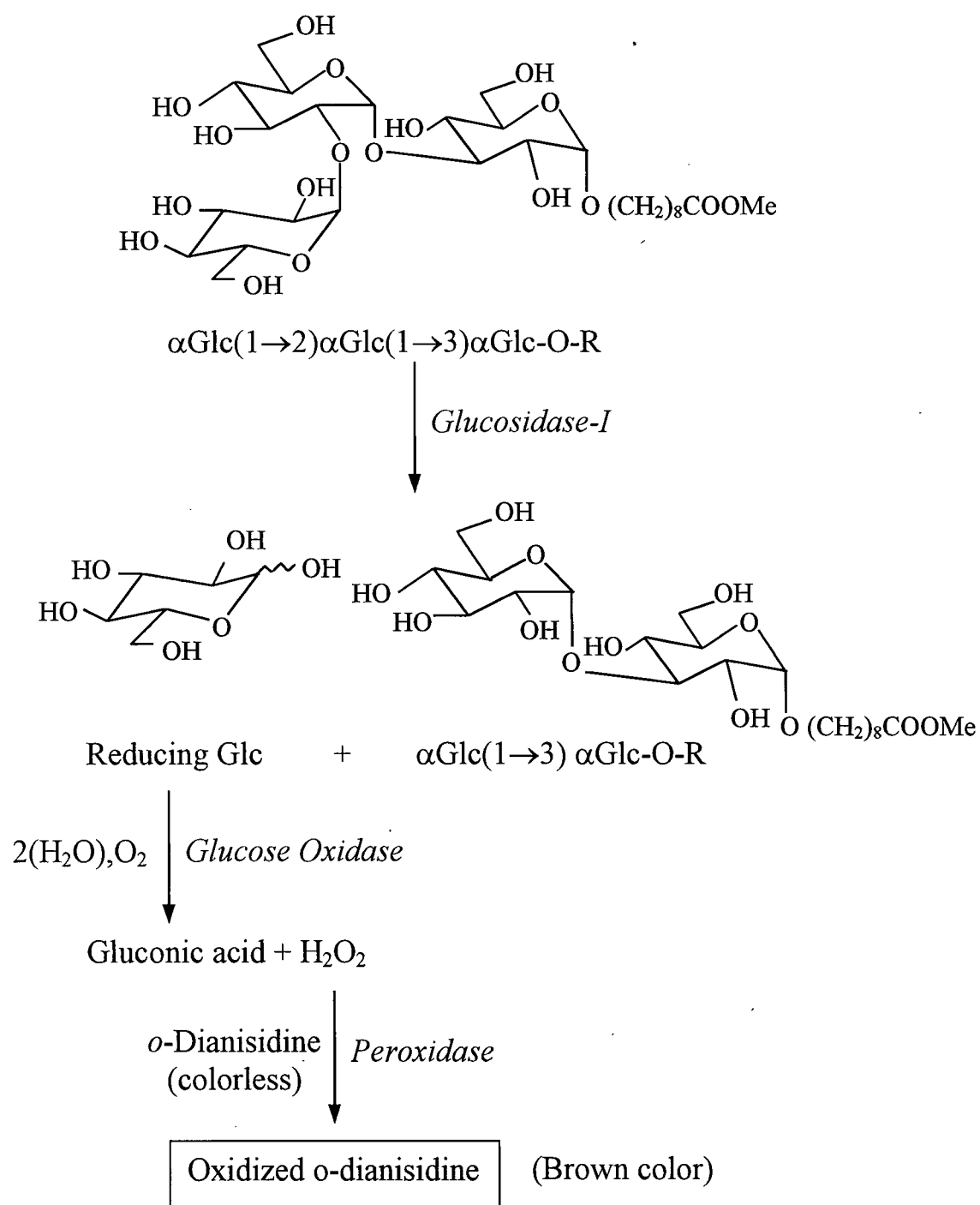
Figure 5. Reaction pathway for glucosidase I activity assay.

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defined as the amount of enzyme required to release 1 nmol glucose/hr at 37°C.

2.1.1.3. Characteristics of Glucosidase I

Based on the enzyme isolated from various sources in these studies, it has been shown that glucosidase I has a pH optimum of 6.2 – 7.0; the enzyme does not require any metal ions for its activity; 1-deoxynojirimycin, a basic analogue of glucose, and its derivatives are potent inhibitors of the enzyme; it is also inhibited by kojibiose and by tris; the enzyme itself is a glycoprotein with high-mannose oligosaccharide; the yeast microsomal enzyme has a subunit with molecular mass of 95 kDa while microsomal enzyme from other sources has a subunit with molecular mass of 85 kDa. The amino acid sequences of glucosidase I from pig liver and human hippocampus were found to have little difference within the coding sequence, demonstrating the close evolutionary relationship between the enzymes from both organisms.

2.1.2. Stabilization of Glucosidase I

Enzymes are relatively fragile substances, with a tendency to undergo inactivation under unsuitable conditions. They are usually exposed to three types of factors which may lead to loss of activity: 1) denaturation; 2) inactivation of catalytic site; and 3) proteolysis (Scopes, 1982). Therefore, in the handling of enzymes, the first consideration must always be to avoid inactivation. Denaturation can be minimized if the principal effectors of denaturation are avoided. These are extremes of pH, temperature, and denaturants such as some organic solvents. The natural pH inside a cell would normally be in the range 6-8, so buffers within this pH range, or as close as possible to the actual pH in the tissue concerned, should protect against pH denaturation. The inactivation of enzymes due to a specific effect

on the catalytic site is more difficult to avoid. Loss of cofactors can be prevented, if they are known, by adding them back or including them in the buffers used. Proteolysis, which is caused by the presence of proteolytic enzymes, can be minimized by mixing proteolytic inhibitors in the buffers.

In purification of glucosidase I, the enzyme was found to be very unstable (Szumilo *et al.*, 1986; Shailubhai *et al.*, 1987). For example, the enzyme lost more than 50% of its activity at room temperature after 24 hours (Shailubhai *et al.*, 1987). Therefore, it is necessary to find a way to stabilize the enzyme in order to further study its characteristics.

2.2. Mechanistic Studies of Glucosidase I

2.2.1. Stereochemistry of Glucosidase I

2.2.1.1. Stereochemistry

There are two major categories of glycosidases. Those that hydrolyze the glycosidic bond with net inversion of configuration are termed inverting enzymes, and those that do so with net retention of anomeric configuration are termed retaining enzymes (Koshland, 1953).

The likely catalytic mechanisms for retaining glycosidases and inverting glycosidases were proposed by Koshland (1953) more than forty years ago and have largely stood the test of time. Retaining glycosidases are generally believed to function through a double displacement mechanism in which a glycosyl-enzyme intermediate is formed and hydrolyzed via oxocarbonium ion-like transition states as illustrated in Fig.6. The reaction is facilitated by acid/base catalysis. It is probable that the same group plays both roles. Inverting glycosidases are believed to function by a single-step mechanism in which a water molecule effects a direct displacement of the glycosidic leaving group from the anomeric center as shown in Fig.7. Again this displacement mechanism is general acid/base catalyzed, but with one active site amino acid acting as the general base, helping to deprotonate the nucleophilic water molecule, and the other amino acid acting as a general acid, protonating the departing oxygen atom in a concerted fashion as the bond cleaves. The reaction proceeds via an oxocarbonium ion-like transition state. This model is supported by 3D structures that are available for several different inverting glycosidases such as the cellulases from *Trichoderma reesei* (Rouvinen *et al.,* 1990) and *Thermomonospora fusca* (Spezio *et al.,*1993), soybean ß-amylase (Mikami *et al.,* 1993), and *Aspergillus awamori* glucoamylase
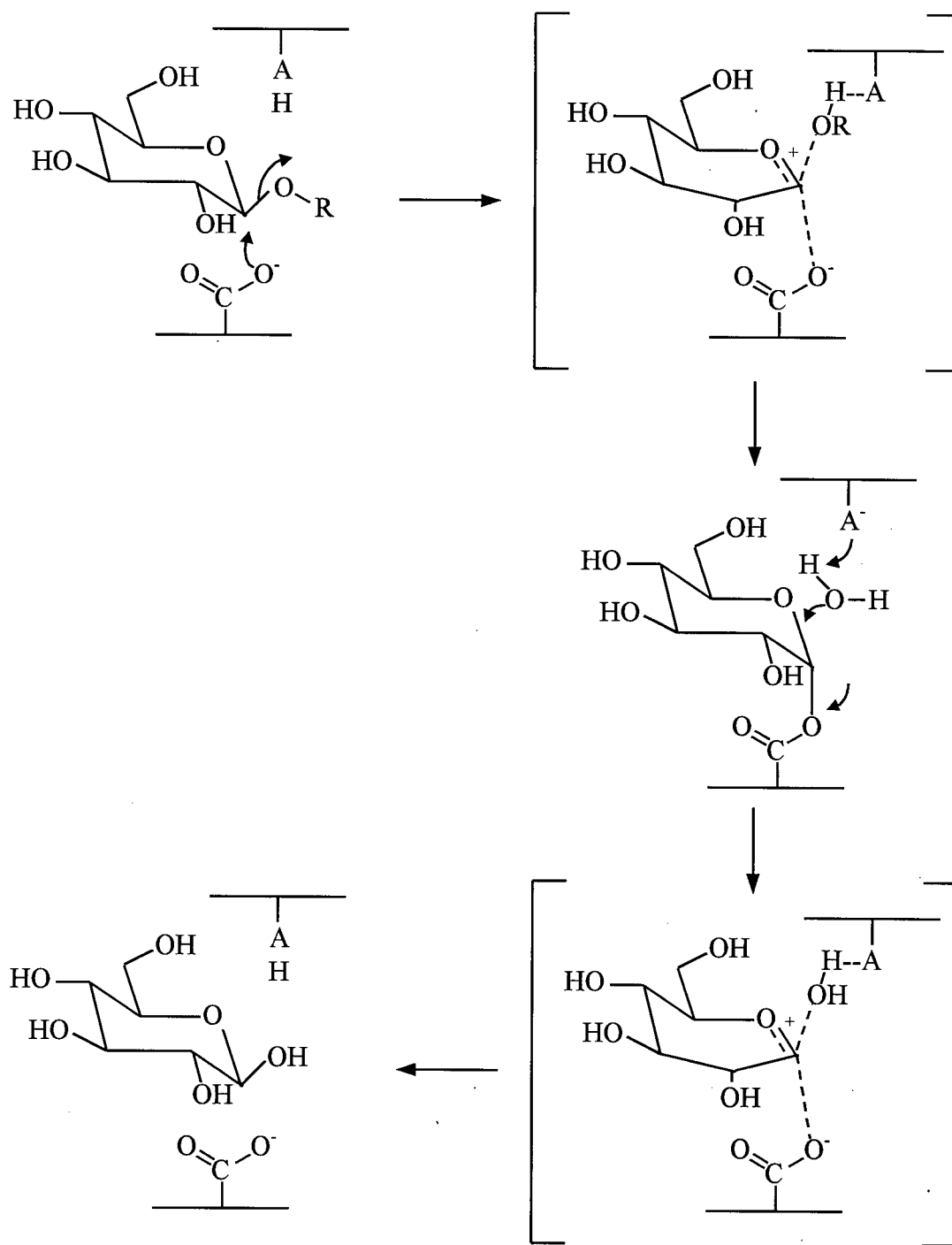
Figure 6. Catalytic mechanism for retaining glycosidases.

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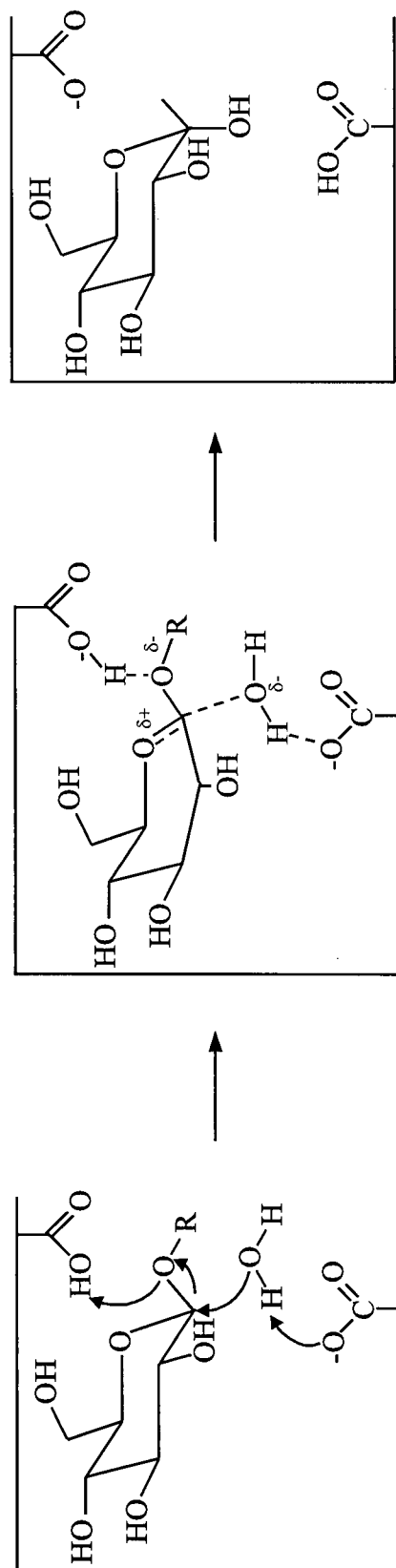
Figure 7. Catalytic mechanism for inverting glycosidases.

(Aleshin *et al.,* 1994). In all cases studied, it is apparent that these acids and bases are the carboxylic side chains of aspartic or glutamic acids.

Although glucosidase I has attracted much attention recently, not many mechanistic or structural studies have been carried out on this enzyme, in part due to difficulties in obtaining large quantities of the natural substrate and the enzyme. As a result, no one has yet been able to place glucosidase I into one of the two categories.

In order to show that if glucosidase I is an inverting enzyme or a retaining enzyme, it is necessary to study the stereochemical course of glucosidase I reaction. As pointed out by Koshland (1953), an enzymatic reaction can be considered as a reaction between organic molecules in which the catalyst happens to be a protein. Such a consideration allows the tools of physical organic chemistry to be applied to the study of the enzyme mechanism. One of the particularly powerful tools used in the elucidation of chemical mechanisms is stereochemistry. Stereochemical analysis has traditionally provided fundamental information about transition states and intermediates in enzymatic as well as nonenzymatic reactions. In enzymatic reactions, stereochemical analysis generates information about both the chemical reaction mechanisms and specific binding interactions between substrates or inhibitors and enzymatic binding sites (Frey, 1986). This information complements mechanistic information obtained in kinetic, spectroscopic, inhibition, and other studies, bringing the reaction mechanism into clearer focus.

In the past two decades, stereochemical course of various enzymatic reactions have been studied and shown to be useful in categorizing enzymes. Among these studies, NMR (Nuclear Magnetic Resonance) spectroscopy was one of the techniques that have been successfully employed. For example, the NMR spectrum led to the conclusion that

staphylococcal nuclease is an inverting enzyme (Hibler *et al.,* 1986).

2.2.1.2. NMR Spectroscopy

NMR is a spectroscopic technique based on the magnetic properties of atomic

nuclei. It is a powerful tool for structural studies. Like electrons, nucleons possess angular

momentum as a result of their intrinsic spin and orbital motion. When several nucleons are

present in a single nucleus, their angular momenta combine to yield a net angular

momentum characteristic of the nucleus as a whole. This angular momentum is known as

the nuclear spin. A particle possessing angular momentum is affected by an external

magnetic field. For a hydrogen atom, since the nucleus is just a proton, which has spin ½,

the application of a magnetic field causes a hydrogen atom to assume one of two different

orientations with respect to the field. As shown in Fig.8, the spin axis may be aligned

parallel to the magnetic field direction or antiparallel to it. The atom has a different energy

corresponding to each of these alignments, and the application of a magnetic field gives rise

to two different quantum states. If electromagnetic radiation with a frequency satisfies the

energy difference between these states on a sample placed in a magnetic field, the radiation

can be absorbed. The absorption associated with the transition between the two states

constitutes the nuclear magnetic resonance. The technique is valuable because the external

magnetic field is modified by the presence of electrons in the vicinity of the absorbing

hydrogen atom. The magnitude of the field required to induce absorption consequently

depends on the specific chemical environment of each hydrogen atom present in a molecule.

Therefore, different molecules will have unique NMR spectrum of their own. For a simple

compound, peaks appearing in the NMR spectrum are singlets. For a more complicated

molecule, hydrogen nuclei can interact with other hydrogen nuclei possessing magnetic

(a)   (b)
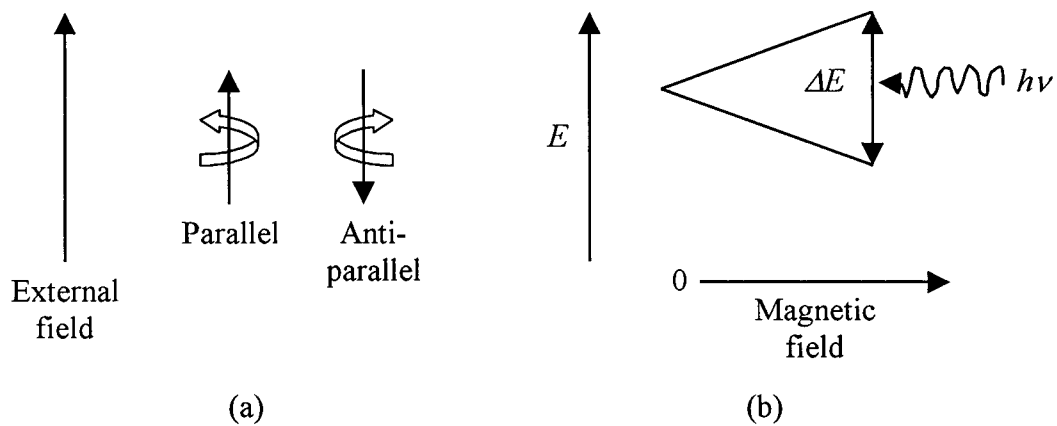
Figure 8. Effect of external magnetic field on a proton. (a) Parallel and antiparallel alignments of the proton spin axis relative to the direction of the field. (b) The proton can occupy one of two quantum states depending on the direction of its alignment. The energy difference ΔE between the states increases with the magnitude of the applied field. In the presence of a magnetic field, electromagnetic radiation with a frequency satisfying the relation $\Delta E = h\nu$ is absorbed and induces a transition between the states (Adapted from Porile, 1987).

moments to cause mutual splitting of the nuclear magnetic resonance peaks into multiplets. The coupling of one set of equivalent spins with another set of spins is termed "spin-spin coupling" or "spin-spin splitting". The magnitude of the splitting is designated by the spin-spin coupling constant J and is independent of the applied magnetic field strength. An unperturbed nucleus or equivalent set of nuclei will give a singlet. Multiplet splitting of a resonance from a set of equivalent nuclei will be determined by nearby sets of equivalent nuclei. There are various nuclear magnetic resonance spectral parameters that are summarized in Fig.9. The NMR spectrum is composed of bell-shaped resonance peaks with the following characteristics: 1) chemical shift (S) - denotes peak position relative to a reference (H_o); 2) amplitude - height of peak in arbitrary units; 3) intensity - integrated area under the peak; 4) linewidth ($W_{1/2}$) - measure of the width of the peak at half maximal amplitude (in Hz); and 5) spin-spin coupling constant (J_{ac})- separation (in Hz) between the peaks in a multiplet. These NMR spectrum parameters provide an indication of the types and relative numbers of groups present, and thus provide a detailed picture of molecular structure. By continuously monitoring the changes of the substrate(s) and product(s) using the NMR spectroscopic technique, the stereochemical course of an enzymatic reaction can be then studied.

2.2.2. *Catalytic Amino Acid Identification at the Active Site*

The different stereochemical outcomes of retaining and inverting glycosidases demand quite different catalytic mechanisms, and thus are likely to have different active site components and structures. There are several different approaches for the identification of catalytic amino acid residues at the active site of glycosidases. These include methods using
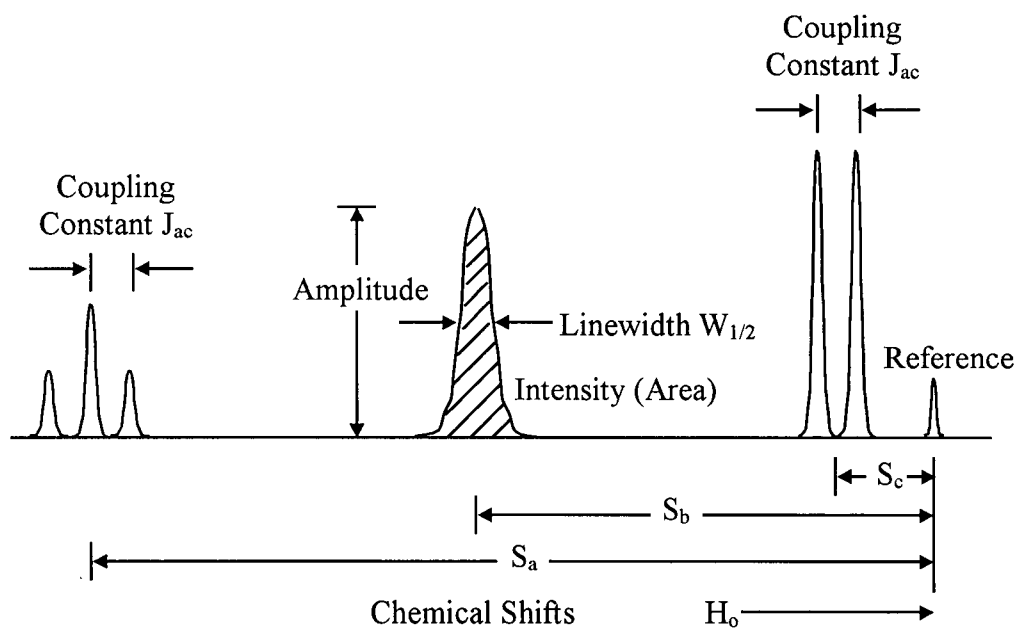
Figure 9. The various nuclear magnetic resonance spectral parameters (Adapted from James, 1975).

sequence alignment, 3D structure analysis, group-specific labels, derivatization with affinity labels, and active site labeling with mechanism-based inhibitors. The pros and cons of these approaches have been reviewed in great detail by Withers and Aebersold (1995).

Group-specific labels are designed to form stable, covalent bonds with specific functional groups located at the active site. Various group-specific reagents have been used in this approach, including Woodward's reagent K and ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDAC) for carboxyl groups (Tomme and Claeyssens, 1989; Bray and Clarke, 1994), phenylglyoxal and 2,3-butandione for arginine, N-bromosuccinimide for tryptophan, tetranitromethane for tyrosine, N-ethylmaleimide for cysteine, and diethylpyrocarbonate for histidine (Romaniouk and Vijay, 1997).

Due to the simplicity of group-specific labels, scientists have started to look at the active site components of glucosidase I with this approach in the past decade. Using DNJM (1-deoxynojirimycin) to protect the active site and various specific reagents to modify the enzyme, it was found that a cysteine (Pukazhenthi *et al.*, 1993), an arginine and a tryptophan residue (Romaniouk and Vijay, 1997) are located at the active site of glucosidase I from bovine mammary gland.

2.2.3. Inhibitors of Glucosidase I

Any substance that decreases the velocity of an enzyme-catalyzed reaction can be considered to be an inhibitor. Enzyme inhibitors are generally classified into three simple categories including competitive inhibitor, noncompetitive inhibitor and uncompetitive inhibitor. A competitive inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding. The inhibitor and the substrate are mutually exclusive,

which is often because of true competition for the same site. The equilibria describing

competitive inhibition can be shown as below:

$$E + S \overset{K_s}{\underset{}{\rightleftharpoons}} ES \overset{K_p}{\longrightarrow} E + P$$
$$+$$
$$I$$
$$K_i \updownarrow$$
$$EI$$
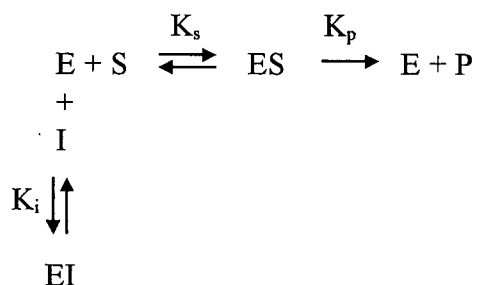
Where $K_i = [E][I]/[EI]$, $K_s = [E][S]/[ES]$, and $K_p =$ rate constant for the breakdown of ES

to $E + P$.

The competition and mutual exclusion of S and I are clearly seen. An increase in [S]

at constant [I] decreases the degree of inhibition. An increase in [I] at constant [S] increases

the degree of inhibition. The lower the value of K_i, the greater is the degree of inhibition at

any given [S] and [I]. Each of the various categories of inhibition has its own characteristic

$1/v$ versus $1/[S]$ plot (Fig.10). For competitive inhibition, a competitive inhibitor acts only

to increase the apparent K_m (measured as [S] required for $\frac{1}{2} V_{max}$) for the substrate. As [I]

increases, Km_{app} increases but the V_{max} remains unchanged. For noncompetitive inhibition, a

classical noncompetitive inhibitor decreases V_{max}, but has no effect on the K_m value. For

uncompetitive inhibition, both Km_{app} and V_{max} will change as [I] changes. V_{max} will be lower

in the presence of an uncompetitive inhibitor than the V_{max} in the absence of an

uncompetitive inhibitor. Unlike noncompetitive inhibition, however, the apparent K_m value

will decrease.

In addition to the above three simple categories of inhibition, there is a more

complicated type of inhibition - mixed type inhibition. As the name suggests, a mixed type

Competitive

$$\frac{1}{V_{\max}} = \frac{1}{V_{\max(I)}}$$

$\dfrac{1}{v}$

$\uparrow$ [I]

$-\dfrac{1}{K_m}$ $-\dfrac{1}{K_{m(I)}}$ 0 $\dfrac{1}{s}$

Non-Competitive

$\dfrac{1}{v}$

$\uparrow$ [I]

$\dfrac{1}{V_{\max(I)}}$

$\dfrac{1}{V_{\max}}$

$-\dfrac{1}{K_m}$ 0 $\dfrac{1}{s}$

$= \dfrac{1}{K_{m(I)}}$

Uncompetitive

$\dfrac{1}{v}$

$\dfrac{1}{V_{\max(I)}}$

$\uparrow$ [I]

$\dfrac{1}{V_{\max}}$

$-\dfrac{1}{K_{m(I)}}$ $-\dfrac{1}{K_m}$ 0 $\dfrac{1}{s}$
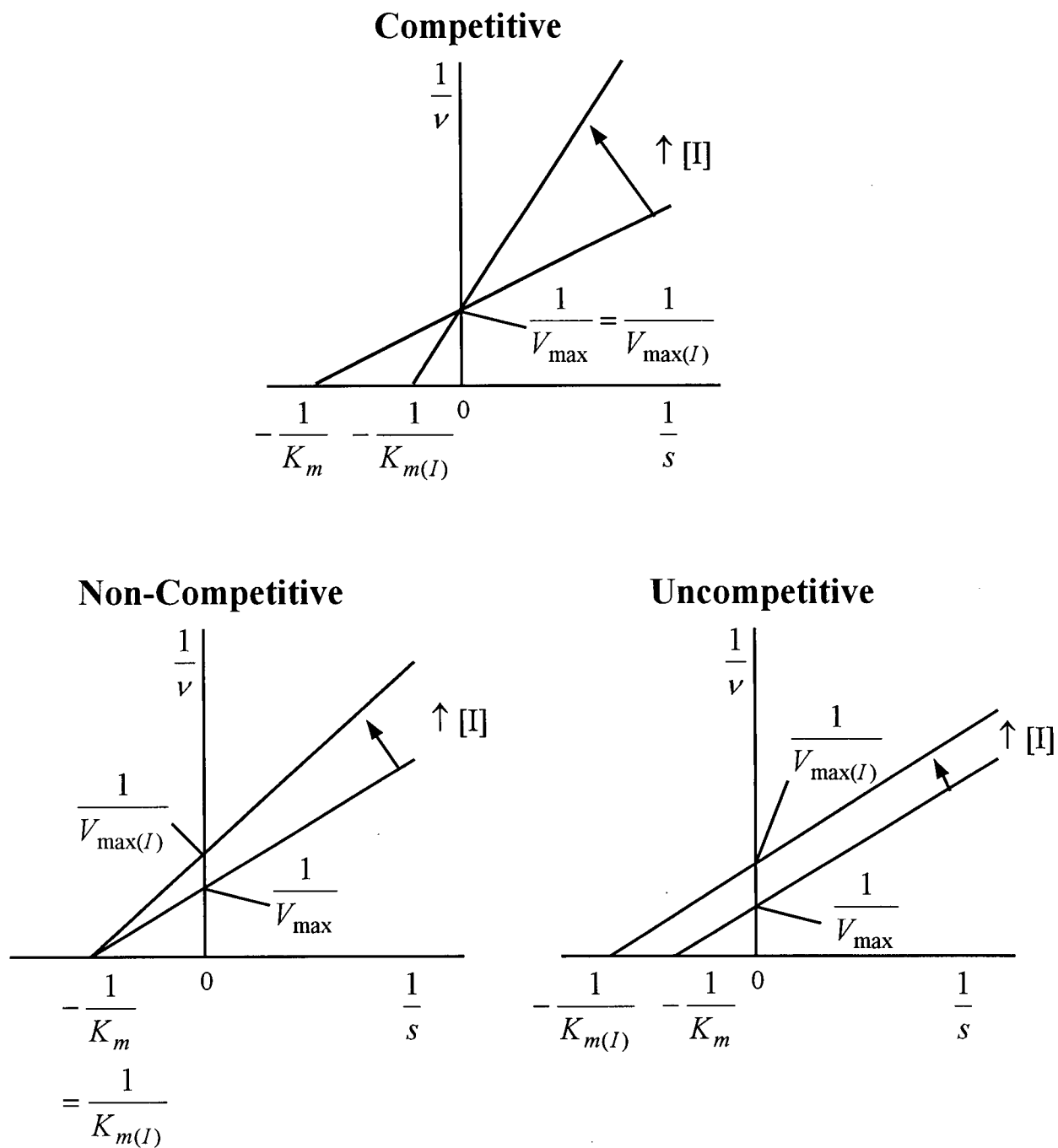
Figure 10. Characteristic relation between $1/v$ and $1/[S]$ for various types of inhibition.

inhibitor affects both the V_{max} and K_m values of an enzyme-catalyzed reaction. The general 1/v versus 1/[S] plot for this type of inhibition is shown in Fig.11.

Inhibitors of glycoprotein processing have been very useful as probes to study the role of N-linked oligosaccharides in glycoprotein function (Elbein, 1991a). Several inhibitors were found to be able to block the modification of N-linked oligosaccharides by inhibiting the glycosidases that remove sugars from the $Glc_3Man_9(Glc-NAc)_2$-protein (Fig.12). Among these compounds, the indolizidine alkaloid castanospermine that is found in the seeds of the Australian tree, *Castanospermum australe* (Szumilo *et al.*, 1986) and the azahexose deoxynojirimycin, found in the culture medium of certain bacteria of the *Streptomyces* or *Bacillus* genera (Niwa *et al.*, 1970), were shown to be potent inhibitors of glucosidase I. Castanospermine achieves 50% inhibition at about 2 or 3 μM while deoxynojirimycin inhibits the enzyme activity by 50% at about 5 to 7 μM. In other studies (Shailubhai *et al.*, 1987), heavy-metal ions including Co^{2+}, Zn^{2+}, Ag^+ and Hg^{2+} strongly inhibited glucosidase I activity. This type of inhibition can be reversed by adding an excess of dithiothreitol. Effect of a number of mono- and di-saccharides were also tested as potential inhibitors (Shailubhai *et al.*, 1987). Among the saccharides tested, only kojibiose, an α-1,2-linked glucose disaccharide, inhibited the enzyme significantly, which supports the postulate that the distal glucose residue is attached on α-1,2-glucosidically to the $Glc_2Man_9GlcNAc_2$ oligosaccharide core.

Both castanospermine and deoxynojirimycin are potent inhibitors of viral replication including the AIDS virus and tumor cell growth in tissue-culture cells (Walker *et al.*, 1988; Montefiori *et al.*, 1988; Gruters *et al.*, 1987). Consequently, several of their derivatives have been tested in clinical trials as possible chemotherapeutic agents (van den Broek *et al.*,
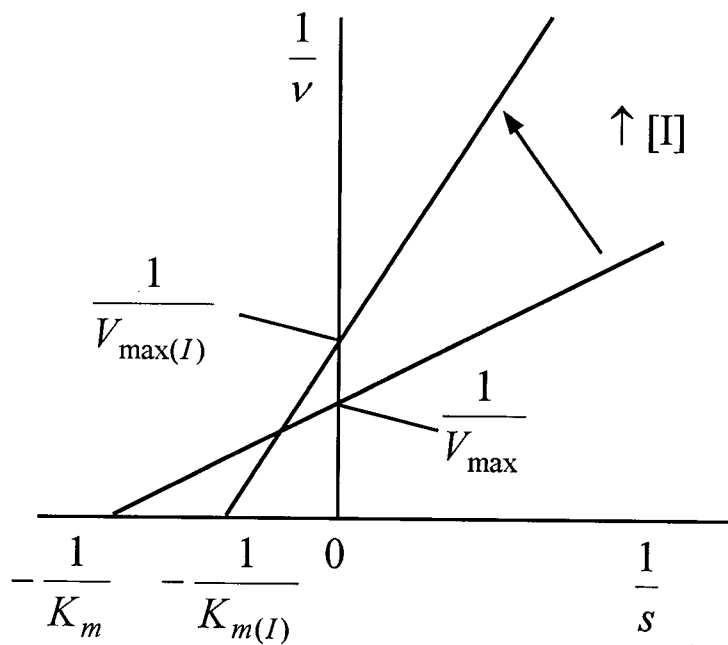
Figure 11. Mixed-type inhibition.

Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol

HO—[Castanospermine structure]
OH
HO
OH
Castanospermine

Man-Man
Man
Man-Man
Man-GlcNAc$_2$-Protein
Glc-Glc-Glc-Man-Man-Man

↓ ⇢ 3 Glc

CH$_2$OH
[Deoxynojirimycin structure]
OH
HO
OH
Deoxynojirimycin

Man-Man
Man
Man-Man
Man-GlcNAc$_2$-Protein→ High
Man-Man-Man
mannose
chains

↓ ⇢ 4 Man

CH$_2$OH
[Deoxymannojirimycin structure]
OHHO
HO
Deoxymannojirimycin

Man
Man
Man
Man-GlcNAc$_2$-Protein
Man

Man
Man
Man
Man-GlcNAc$_2$-Protein→ Hybrid
GlcNAc-Man
chains

↓ ⇢ 2 Man

OH
[Swainsonine structure]
OH
N
OH
Swainsonine

Man
Man-GlcNAc$_2$-Protein
GlcNAc-Man

↓
↓
↓

Complex chains

Figure 12. Site of action of various inhibitors that act on the glucosidases involved in the processing pathway.

30

1996).

3. Methods and Materials
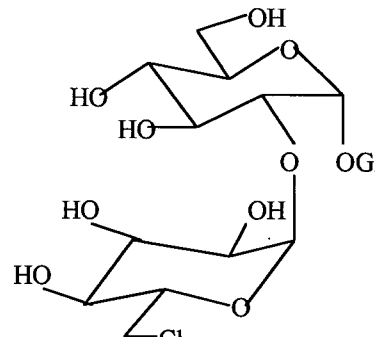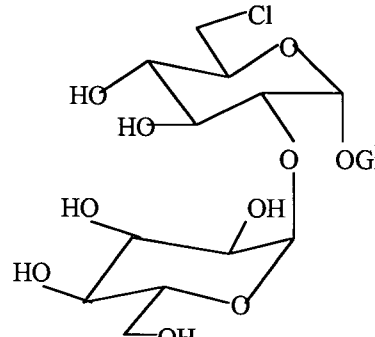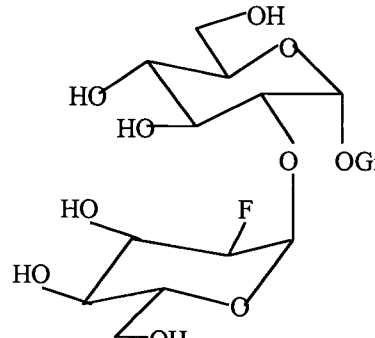
3.1. Materials
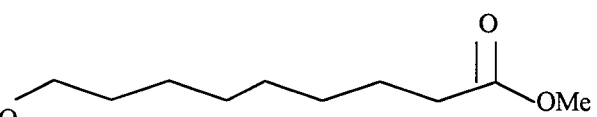
Autolyzing yeast cells (*Saccharomyces cerevisiae*), phenylmethylsulfonyl fluoride (PMSF), p-nitrophenyl-alpha-D-glucopyranoside (PNPG), protease inhibitor cocktail [containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane(E-64), and 1,10-phenanthroline], 1-deoxynojirimycin (DNJM), Concavalin A, sepharose, α-methylmannoside, α-methylglucoside, thioglycolate, CAPS transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid], ß-mercaptoethanol, bromophenol blue, ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) (obtained from Sigma-Aldrich Canada Ltd, Oakville, ON); monobasic sodium phosphate (NaH_2PO_4), glycerol, ammonium sulfate, Tris, sodium carbonate, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), $MgCl_2$, $MnCl_2$, $CaCl_2$, methanol, ethanol, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), glycine, N,N,N',N'-tetramethylethylenediamine (TEMED), cupric acetate (obtained from Fisher Scientific, Fair Lawn, NJ); bovine serum albumin (BSA), ammonium persulfate (APS), coomassie blue, polyvinylidene difluoride (PVDF) protein sequencing membrane, broad range SDS-PAGE standard (obtained from Bio-Rad Laboratories, Richmond, CA); NaCl, acetic acid, isopropanol (obtained from BDH Inc., Toronto, ON); synthesized trisaccharide substrate [α-D-Glc1$\rightarrow$2α-D-Glc1$\rightarrow$3α-D-Glc-O(CH$_2$)$_8$COOCH$_3$] and various substrate analogues (see Table 2) (obtained from Dr. Om Srivastava at the Alberta Research Council, Edmonton, AB). All electrophoresis reagents were electrophoresis grade. All other chemicals used were reagent grade.

Table 2. Structures of substrate analogues.

Compound	Structure
αGlc(1-2)αGlc(1-3)αGlc-O-grease	
3'Deoxy-αGlc(1-2)αGlc-O-grease	
4'Deoxy-αGlc(1-2)αGlc-O-grease	
6'Deoxy-αGlc(1-2)αGlc-O-grease	

2'O-MethylαGlc(1-2)αGlc-O-grease	
4'O-MethylαGlc(1-2)αGlc-O-grease	
2'NH₂-αGlc(1-2)αGlc-O-grease	
2'N₃-αGlc(1-2)αGlc-O-grease	

6'Chloro-αGlc(1-2)αGlc-O-grease	
6Chloro-αGlc(1-2)αGlc-O-grease	
2'Fluoro-αGlc(1-2)αGlc-O-grease	

For all the structures above   OGr =

35

3.2. Purification of Soluble Glucosidase I

3.2.1. Isolation of Soluble Glucosidase I

The protocol for isolation of soluble glucosidase I was modified from a previous study (Neverova et al., 1994). A 25 g sample of dry yeast cells was suspended in 200 ml of 10 mM phosphate buffer containing 10% glycerol and 100 μM PMSF, pH 6.8 (buffer A), and passed through a French press twice at 12,000 psi. Cell debris was removed by centrifugation at 8,000 x g for 30 min. The supernatant was centrifuged at 200,000 x g for 45 min. Soluble glucosidase I was precipitated out from a 20 - 60% ammonium sulfate fraction of the resulting supernatant. The precipitate was dissolved in 10 ml of buffer A and dialyzed against 2 L of buffer A overnight at 4°C. Dialyzed extract was applied to a Toyopearl DEAE column (2.5 x 20 cm) equilibrated with the same buffer. The column was washed with buffer A containing 0.1 M NaCl. After the protein peak had eluted, glucosidase I was eluted with buffer A containing 0.3 M NaCl. This fraction was dialyzed against 4 L of buffer A at 4°C for 3 hours and then subjected to FPLC with a Source Q column (0.5 x 5 cm). The column was washed with buffer A containing 0.2 M NaCl, and the enzyme was eluted with a linear gradient of 0.2 to 0.5 M NaCl, at a flow rate of 0.20 ml/min with 1-ml fractions collected. Eighty ml of eluant was collected during a typical chromatographic run. Fractions containing glucosidase I were pooled, and stored at 4°C.

3.2.2. Assay of Glucosidase I Activity

The glucosidase I enzyme activity was assayed with a synthetic trisaccharide substrate, α-D-Glc1→2α-D-Glc1→3α-D-Glc-O(CH$_2$)$_8$COOCH$_3$, as described in Neverova et al. (1994). Reactions were initiated by the addition of 4 μl enzyme solution to 1 μl of 10

mM α-D-Glc1$\rightarrow$2α-D-Glc1$\rightarrow$3α-D-Glc-O(CH$_2$)$_8$COOCH$_3$ in 500-μl microfuge tubes.

Tubes were vortexed, briefly microfuged, and incubated at 37°C for 1 hour. The tubes were

again briefly microfuged and the reaction was quenched with Tris, using 25 μl of 1.25 M

Tris-HCl, pH 7.6. The reaction solution was then transferred to a well on a microassay plate

and 20 μl of buffer A was added, followed by 250 μl of combined color reagent, containing

glucose oxidase (5 units/ml), horseradish peroxidase (1 purpurogallin unit/ml) and o-

dianisidine dihydrochloride (40 μg/ml). The solutions were protected from light by covering

with aluminum foil and left to develop for 30 min at 37°C. Absorbances were read at 450

nm with a microplate reader and the amount of glucose released was calculated from the

glucose standard curve. The reaction scheme for the assay is shown in Fig.5. One unit of

activity was defined as the amount of enzyme required for the release of 1 nmol glucose/hr

at 37°C. The activity assay was done on collected fractions at each step during isolation and

the activity, total activity, and specific activity of each fraction were calculated.

Glucosidase II activity and other glucosidase activities were also determined for

each fraction collected during the isolation. This was done by incubating 5 μl of 20 mM p-

nitrophenyl-alpha-D-glucopyranoside and 15 μl of enzyme fraction in a well on a

microassay plate at 37°C for 5 min to 1 hour. Reactions were terminated by the addition of

200 μl of 0.2 M sodium carbonate. The absorbance of each well at 405 nm was read with a

microplate reader. The amount of enzyme required to release 1 nmol glucose per hour was

used to define the enzyme activity.

3.2.3. Protein Assay

The protein content of each fraction collected during isolation steps was determined

according to the standard instructions of the Bio-Rad Protein Assay (standard procedure for microtiter plates). At first, dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts DDI water and filtered through a Whatman #1 filter to remove particulates. Five dilutions of BSA standard were then prepared. Protein solutions were normally assayed in triplicate. Ten microliters of each standard and sample solution was pipetted into separate microtiter plate wells and 200 µl of diluted dye reagent was added to each well. Sample and reagent were then mixed thoroughly, clean tips were replaced and reagent was added to the next set of wells. The microtiter plate was incubated at room temperature for 15 minutes. Finally, absorbance was measured at 595 nm with a microplate reader.

3.2.4. Stabilization of Glucosidase I

The stability of glucosidase I in solution is very poor, especially in the early stages of the enzyme isolation. To try to improve both the yield and long term stability of the enzyme, various additives were added to the phosphate buffer used for extraction and isolation. These additives are different protease inhibitors, including protease inhibitor cocktail [Sigma, product No. P8215, containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane(E-64), and 1,10-phenanthroline] (1%) and PMSF (100 µm); reducing agent - DTT (0.5 mM); chelating agent - EDTA (0.2 mg/ml); polyol - glycerol (10%); and protective protein solution - BSA (1 mg/ml). The levels of concentrations used were based on studies on various enzymes, especially on different glycosidases.

Phosphate buffer was used to control the pH. It can stabilize many enzymes

(Scopes, 1982). The protease inhibitor cocktail, PMSF and EDTA were chosen because they could inhibit various proteolytic enzymes present during the isolation steps which would hydrolyze glucosidase I. The other additives were included in the present study because they have been used during the isolation of various enzymes to help retain enzyme activity. DTT prevents the enzyme from being oxidized; glycerol is a protein stabilizer since it can form strong hydrogen bonds with water, effectively lowering the water activity; and BSA can act as an alternative substrate for proteases which may be present (Scopes, 1982).

Various combinations of additives used during the isolation of the enzyme are listed in Table 3. At different time intervals over the period of 30 days, the activity of isolated glucosidase I was assayed. The enzyme activity was then compared to the activity of the control (isolated with 10 mM phosphate buffer only).

Since it has been mentioned that keeping an enzyme in the deep-freeze state can prolong the half life of the enzyme, or drying an enzyme in high vacuum at low temperature from the frozen state can give an active and soluble powder which can be stored at room temperature (Dixon and Webb, 1964), the effects of freezing (at –25°C and –80°C) and freeze drying treatments on the stability of glucosidase I were also studied in this research.

For freeze drying treatment, aliquots (4 µl) of glucosidase I were stored at –25°C after being freeze dried (medium drying rate, Speed Vac, Model SC 110). Enzyme activity was assayed at different time intervals. Each aliquot was rehydrated with 4 µl of distilled water before the activity assay. For freezing treatment, aliquots of 4 µl of glucosidase I were stored in 500 µl microfuge tubes at –25°C and –80°C respectively and then the enzyme activity was assayed at different time intervals.

It is possible that proteases arising from contaminating bacteria were responsible for

Table 3. Combination of additives used to stabilize glucosidase I during isolation and storage.

Combination	A	B	C	D	E	F	G
10 mM phosphate buffer	x	x	x	x	x	x	x
1% protease inhibitor cocktail		x					
10% glycerol			x	x	x	x	x
100 µM PMSF				x	x	x	x
0.2 mg/ml EDTA					x	x	
1 mg/ml BSA						x	x

the loss of glucosidase I activity. Accordingly, the stability of sterile and non-sterile glucosidase I activity over a period of 30 days were also compared. Isolated glucosidase I (using Combination D, i.e. 10 mM phosphate buffer, 10% glycerol and 100 μM PMSF) was sterilized by passing through a 0.22 μm sterile Millipore Filter Unit into a sterile 50-ml Falcon tube. Aliquots (4 μl) of sterile glucosidase I were aseptically transferred into microfuge tubes and stored at 4°C. All microfuge tubes and pipette tips used for the transfer were previously autoclaved. Non-sterile glucosidase I from the same isolation batch was stored in non-sterile microfuge tubes at 4°C as well. The activity of both sterile and non-sterile glucosidase I was assayed at different time intervals and compared.

3.2.5. Highly Purification of Glucosidase I with Affinity Chromatography

Taking advantage of the high specificity of affinity chromatography, inhibitor ligand affinity chromatography and Con A chromatography were attempted to further purify glucosidase I in this research work in order to study the amino sequence of glucosidase I.

3.2.5.1. Affinity Chromatography with an Enzyme Inhibitor as the Ligand

Synthesis of the affinity ligand, N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, and the coupling of the affinity ligand to AH-Sepharose 4B were done by a collaborating laboratory, according to the procedure in Bause *et al.* (1991).

After glucosidase I was isolated from yeast cells, as described above, 9.5 ml of the enzyme solution was added to 10 ml of packed gel washed with buffer A in a 50-ml falcon tube. The mixture was tumbled at 4°C overnight. After brief centrifugation, the supernatant was removed and assayed for glucosidase I activity. The gel was decanted into a column and washed with 200 ml of 100 mM phosphate buffer at pH 6.8, followed by 200 ml of 10

mM phosphate buffer at pH 6.8. The washed gel was transferred to a 50-ml falcon tube and tumbled overnight at 4°C with 10 ml of 10 mM deoxynojirimycin (DNJM). The supernatant was then concentrated to 50 μl by a 10 K Centricon concentrator (Amicon) and washed twice with 500 μl of phosphate buffer. The final concentrated liquid (purified glucosidase I) was assayed for glucosidase I activity and run on a 10% SDS-PAGE gel.

3.2.5.2. Concanavalin A-Sepharose Chromatography

Pooled enzyme fractions after FPLC step were loaded into a 10 ml Concanavalin A-sepharose column which had been equilibrated with buffer A containing 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$ and 0.5 M NaCl. Then, 10 ml of 1 M α-methylmannoside or α-methylglucoside in buffer A with 0.2 mg/ml EDTA was applied to the column, the column was stoppered and stored at 4°C overnight in contact with the α-methylmannoside or α-methylglucoside. The next day, the elution was continued and additional amounts of α-methylmannoside or α-methylglucoside were passed through the column. The first 40 ml of elution fluid was collected in 40 1-ml fractions and fractions with glucosidase I activity were pooled. The pooled enzyme fractions were then concentrated to 50 μl by a 10 K Centricon concentrator and washed with 500 μl of phosphate buffer twice. The final concentrated liquid (purified glucosidase I) was assayed for glucosidase I activity and run on a 10% SDS-PAGE gel.

Sequencing of glucosidase I purified from Con A-Sepharose chromatography was attempted. The purified enzyme fraction was run on a SDS-PAGE gel (with 10% resolving gel). While the enzyme fraction was being electrophoresed, a sheet of polyvinylidene difluoride (PVDF) membrane was wetted with 100% methanol and equilibrated with CAPS transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH

11.0]. After electrophoresis was completed, the gel was assembled into a blot transfer cassette and the enzyme protein was electroblotted for 6 hours at 30 volts. The PVDF membrane was then washed with two changes of CAPS transfer buffer and stained with 0.1% Coomassie blue in 50% methanol for 5 min. The blot was destained with 50% methanol for 10 min. The membrane was then washed with several changes of distilled-deionized water for 5-10 min and air dried. The stained blot was stored dry at −25°C. The band corresponding to glucosidase I was cut from the membrane and excess membrane carefully trimmed away to give a 2x4 mm segment. The segment was sent for amino sequencing analysis at the Nucleic Acid and Protein Service (NAPS) unit of the Biotechnology Laboratory, UBC.

3.3. Mechanistic Studies of Glucosidase I

3.3.1. Stereochemistry of Glucosidase I Reaction

Glucosidase I was isolated as previously described (using Combination D) and exchanged into 50 mM deuterated sodium phosphate buffer, pD 7.1 (where pD is the uncorrected pH meter reading) using Centricon 10 units (Amicon). The enzyme was then assayed for activity and the stereochemical course of the glucosidase I reaction was studied using H^1-NMR spectroscopy.

H^1-NMR spectra were acquired at 27.0°C on a Varian Inova 600 spectrometer using VNMR 5.3B software. Chemical shifts were reported relative to 0.1% external acetone at 2.225 ppm. After initial acquisition of the reference enzyme solution spectrum (700 µl in 5 mm NMR tubes), 60 µl of aliquots of concentrated trisaccharide substrate were added in to give a final concentration of 9.3 mM. A spectrum was recorded after 3 min, then every 5 min for 2.5 hr and finally every 30 min until hydrolysis was complete. In this way the reaction's progress was monitored for up to 15 hr. Spectra were processed with a Gaussian window function of width *at/4* and shifted by *at/6*. A first order baseline correction was applied prior to data analysis, integration and plotting. For integrations, the H^1 signal that remained unchanged over the course of the reaction, was used as a reference.

3.3.2. Kinetic Evaluation of Glucosidase I Substrate Analogues

Since inhibition studies of glucosidase I could be beneficial in preventing or curing various human diseases including AIDS and cancer, further exploration of the possible inhibitory mechanism of this enzyme was done by evaluating the kinetic effects of some synthesized glucosidase I substrate analogues. The glucosidase I substrate was αGlc(1-

2)αGlc(1-3)αGlc-O-(CH$_2$)$_8$COOCH$_3$ which was first used by Neverova *et al.* (1994).

Substrate analogues including 3'Deoxy-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 4'Deoxy-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 6'Deoxy-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 2'O-Methyl-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 4'O-Methyl-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 2'NH$_2$-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 2'N$_3$-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 6'Chloro-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$, 6Chloro-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$ and 2'Fluoro-αGlc(1-2)αGlc-O-(CH$_2$)$_8$COOCH$_3$ were synthesized by Dr. Om Srivastava at the Alberta Research Council, Edmonton, Alberta. Structures of these substrate analogues can be found in Table 2.

Prior to a full kinetic evaluation, glucosidase I activity of the pooled enzyme fractions from FPLC in the presence of different substrate analogues (1 and 10 mM) was monitored and compared to the activity when such analogue compounds were not added by calculating the percentage enzyme activity remaining.

For substrate analogues that were found to have relatively potent inhibitory effect on glucosidase I, a full kinetic evaluation of the mechanism of inhibition was determined. The initial rate data obtained at three different concentrations of the compound over a range of substrate concentrations (0.75, 1.0, 2.0, 4.0, 7.5, 10.0, 12.5 and 15.0 mM) and fit to the Michaelis-Menten equation, using nonlinear regression analysis employing the Marquardt-Levenberg algorithm (SigmaPlot, Jandel Scientific, 1991).

3.3.3. Site-Specific Chemical Modification of Glucosidase I

3.3.3.1. Inhibitory Effect of EDAC on Glucosidase I

To determine if there is an acidic amino acid (carboxyl group containing amino acid)

present at the active site of glucosidase I, the effect of the carboxyl group specific reagent, ethyl-3-[3-(dimethylamino)propyl]carbodiimide - EDAC, on glucosidase I activity was examined. EDAC was made up into different concentrations (5, 10, 20, 30, 40, and 50 mM) with pH at 6.8. At each concentration, EDAC was tested to determine whether there was inhibitory effect on glucosidase I activity with various incubation times (30, 45, and 60 min), as described below.

A 4 µl aliquot of EDAC (0, 5, 10, 20, 30, 40, 50 mM) was added into a 500-µl microfuge tube and lyophilized. The microfuge tube was re-constituted with 4 µl of enzyme solution and incubated at 4°C for certain amount of time (30, 45, 60 min). Enzyme activity was then measured using the activity assay and compared to activity of the control (EDAC at 0 mM). A flow chart of the detailed procedure is shown in Fig.13. The experiments were done in duplicate. In addition, the background absorbance at 450 nm by EDAC, buffer A and H_2O was negligible, and EDAC was found not to inhibit the coupling enzymes in the glucosidase I activity assay.

3.3.3.2. Test for Active Site Components of Glucosidase I

10 mM of DNJM was used to protect the active site of glucosidase I (2 µl of DNJM at 40 mM in a 500-µl microfuge tube was lyophilized and re-constituted with 8 µl of the enzyme solution), followed with the addition of 50 mM EDAC (transferred the 8 µl of mixture of DNJM and enzyme solution into a 500-µl microfuge tube in which 2 µl of EDAC at 200 mM had been lyophilized). After removing the DNJM and excessive EDAC by dialysis, activity of the modified enzyme was determined and compared to the activity of enzyme treated with 50 mM EDAC only. Controls without being treated with DNJM and/or EDAC also underwent the same experimental procedure, i.e. with equal time period for

4 μl of EDAC (0, 5, 10, 20, 30, 40, 50 mM) lyophilized in 500-μl microfuge tubes

+ 4 μl of enzyme solution

Incubated at 4°C for 30, 45, 60 min

+ 1 μl of trisaccharide

Incubated at 37°C for 1 hr

+ 25 μl of 1.25 M Tris-HCl, pH 7.6

+ 20 μl of buffer A

Transferred to a microassay plate

+ 250 μl of combined color reagent

Incubated at 37°C in dark for 30 min

Absorbances read at 450 nm

Activity compared with controls (without EDAC)

Figure 13. Procedure of EDAC inhibitory effect on glucosidase I.

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procedure is outlined in Fig.14. Briefly, three sets of glucosidase I samples were prepared. The first set of samples were treated with DNJM and later with EDAC. The second set of samples were treated with EDAC only. The third set of samples were used as controls without being treated with DNJM or EDAC. Glucosidase I activity assay was carried out for all three sets of samples at each step during the test. The enzyme activity was recorded and compared. The experiments were done in duplicate and DNJM was found not to affect the glucosidase I activity assay.
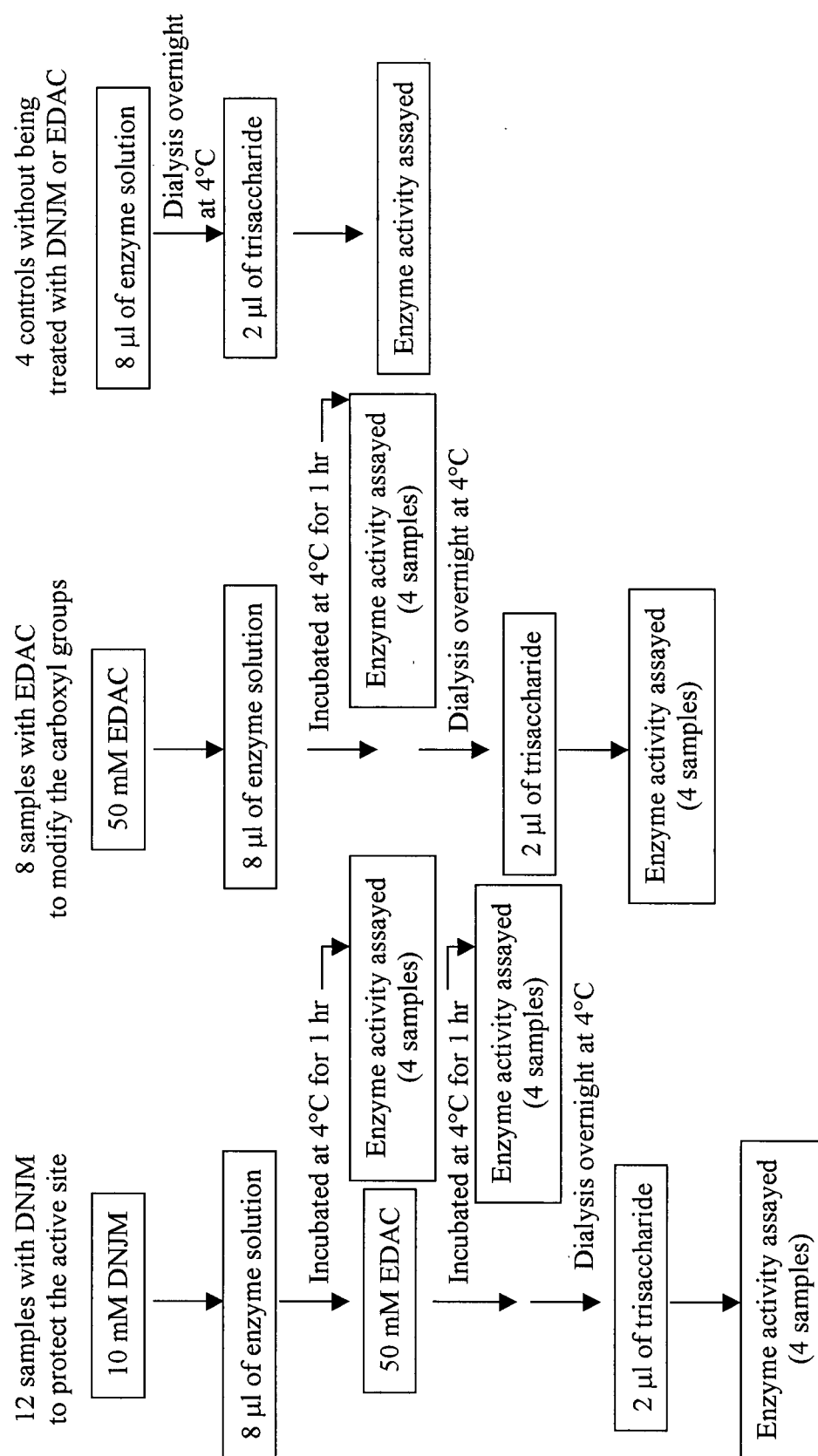
Figure 14. Procedure for the site-specific chemical modification of glucosidase I.

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4. Results and Discussion

4.1. Isolation of Glucosidase I

Dry yeast cells were chosen as the source for soluble glucosidase I since they are readily available and have been successfully used elsewhere (Neverova *et al.*, 1994). The isolation of the soluble enzyme was followed with a synthetic trisaccharide α-D-Glc(1→2) α-D-Glc(1→3)α-D-Glc-O(CH$_2$)$_8$COOCH$_3$ as substrate. The terminal glucose is released from the substrate by the enzyme and quantitated using glucose oxidase, peroxidase, and o-dianisidine. The enzyme was isolated by a three-step procedure involving ammonium sulfate extraction, Toyopearl DEAE chromatography, and FPLC with a Source Q column. The results of a typical isolation experiment are summarized in Table 4.

Crude glucosidase I protein was precipitated with a 20 – 60% ammonium sulfate fraction of the yeast cell supernatant after the ultracentrifugation step and dialyzed against 2 L of buffer A overnight. This fraction of crude protein had a much higher protein concentration and lower glucosidase I specific activity compared to enzyme fractions obtained from later isolation steps, indicating high content of contaminating proteins. This can also be partly shown by the high glucosidase II activity of this crude protein fraction. The high content of impurities resulted in a high background microplate reading of the sample blank of 0.142 AU, compared to a reading of 0.065 AU for the sample blank after FPLC, when assaying the glucosidase I activity, which might have underestimated the enzyme activity of this crude protein fraction since the enzyme activity was calculated based on relatively small absorbance differences in microplate readings between control and sample. This may explain why the total activity of this crude protein fraction is less than that of other enzyme fractions in Table 4. Another possibility is that some of the H$_2$O$_2$ being

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Table 4. Isolation of soluble glucosidase I (from 25 g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	G-I Activity			G-II Activity (nmol/µl of enzyme*hr)
			Activity (nmol/µl of enzyme*hr)	Total activity (µmol/hr)	Specific activity (nmol/µg*hr)	
Dialyzed crude Protein (just before DEAE)	23	22.69	0.531	12.22	0.023	55.475
0.3M NaCl Elution	54	1.34	0.502	27.11	0.375	0.525
Dialyzed protein (just before FPLC)	54	1.68	0.433	23.38	0.258	0.458
G-I collected	10	0.91	1.169	11.69	1.284	0.102

produced was degraded by some other oxidoreductases so that glucosidase I activity was underestimated by the enzyme assay in the crude mixture. Chromatography of the crude protein fraction on a Toyopearl DEAE column increased the purity of the enzyme protein by approximately 10-fold. This step was very critical since it separated glucosidase I from the majority of glucosidase II and other non-specific α-glucosidase activities. The glucosidase II activity (or PNPG activity) dropped approximately 100-fold from the previous step. After three-hour dialysis against 4 L of buffer A, the glucosidase I activity decreased by about 15% which shows that enzyme glucosidase I is very unstable. After this enzyme fraction was subjected to FPLC with a Source Q column, the specific activity of glucosidase I increased approximately by 3 - 4 times from the fraction obtained from the Toyopearl DEAE column. Therefore, this is also an important step since it further decreased the glucosidase II activity by several-fold while increasing the glucosidase I specific activity by several-fold from the previous step. The ratio of glucosidase I / glucosidase II activity after FPLC was around 12 / 1. Throughout this research, all glucosidase I used for other studies, with the exception of stabilization study where the enzyme was isolated with Combinations A – G (to be discussed in the next section), was isolated using this procedure (Combination D as in the stabilization study). Variation of enzyme activity at each isolation step during each preparation ranged from approximately 3 to 8%.

Autolyzing the yeast cells by incubating the yeast cell solution at 37°C for 3 hours instead of passing the yeast cell solution through a French press twice was also tried. However, enzyme isolated with this method had lower activity by 20% to 32% compared to enzyme isolated with a French press.

4.2. Stabilization of Glucosidase I

Since glucosidase I activity is measured through a series of chemical reactions (Fig.5), it is critical to ensure that the coupling enzymes and components are not affected by any additives or conditions used. Otherwise, the glucosidase I activity measured will not be very accurate. The effect of the various combinations of additives used to stabilize glucosidase I activity on the enzyme coupled assay was therefore investigated. The results indicated that DTT interfered with the assay (Table 5). This is because the H_2O_2 produced was reduced by DTT masking the glucosidase I activity. DTT was therefore, not used as an additive in further work.

Total activity and percentage yield of glucosidase I isolated with various combinations of additives that could protect the enzyme from inactivation are summarized in Table 6. The results indicated that the total activity of glucosidase I was greatly improved by the use of different additives. The data suggested that the protease inhibitor cocktail, glycerol, PMSF, EDTA and BSA could all help to increase the total yield of glucosidase I. Detailed results including volume, protein content, and enzyme activity of each fraction during isolation steps are in Tables (A - G) in Appendix 1.

The effect of different combinations of additives on the stability of glucosidase I was tested by storing the enzyme at 4°C over a period of 30 days, a reasonable time period to determine the trend of enzyme activity loss, and examining the enzyme activity at various times during storage. Among the different trials that were done, glucosidase I isolated with a combination of 10 mM phosphate buffer, 10% glycerol, 0.2 mg/ml EDTA, 100 μm PMSF, and 1 mg/ml BSA (Combination F) had the best stability, while glucosidase I isolated with 10 mM phosphate buffer only (Combination A) was the least stable (Fig.15).

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Table 5. The interference of various additive solutions with the glucosidase I assay.

Solutions	A^c	B	C	D	E	F	G	H^d	EDAC	DNJM
$\dfrac{\Delta AU_{450}1^a}{\Delta AU_{450}2^b}$	1.00	0.96	0.97	0.98	0.94	0.95	0.98	0.09	0.95	0.99

^{a} $\Delta AU_{450}1 = AU_{450}$ of (1 µl of std glu + 24 µl of solution + 25 µl of Tris-HCl + 250 µl of color reagent in G-I assay)

     $- AU_{450}$ of (1 µl of H_2O + 24 µl of solution + 25 µl of Tris-HCl + 250 µl of color reagent in G-I assay)

     = Absorbance caused by 1 µl of std glu when additives are present

^{b} $\Delta AU_{450}2 = AU_{450}$ of (1 µl of std glu + 24 µl of H_2O + 25 µl of Tris-HCl + 250 µl of color reagent in G-I assay)

     $- AU_{450}$ of (25 µl of H_2O + 25 µl of Tris-HCl + 250 µl of color reagent in G-I assay)

     = Absorbance caused by 1 µl of std glu when additive are not present

^{c} A to G = 10 mM phosphate buffer with various additives, as A to G defined in Table 3

^{d} H = D + 0.5 mM DTT

Table 6. Total activity and % yield of glucosidase I isolated from 25 g of dry yeast cells with different combination of additives.

Combination	Total Activity (mmol/hr)	Yield (%)[a]
A	0.13	10.32
B	0.23	23.23
C	0.22	22.92
D	0.47	43.52
E	0.48	52.75
F	0.73	76.04
G	0.54	54.55

[a] Yield (%) is calculated as units of activity from FPLC / units of activity of 0.3 M NaCl/Toyopearl DEAE.
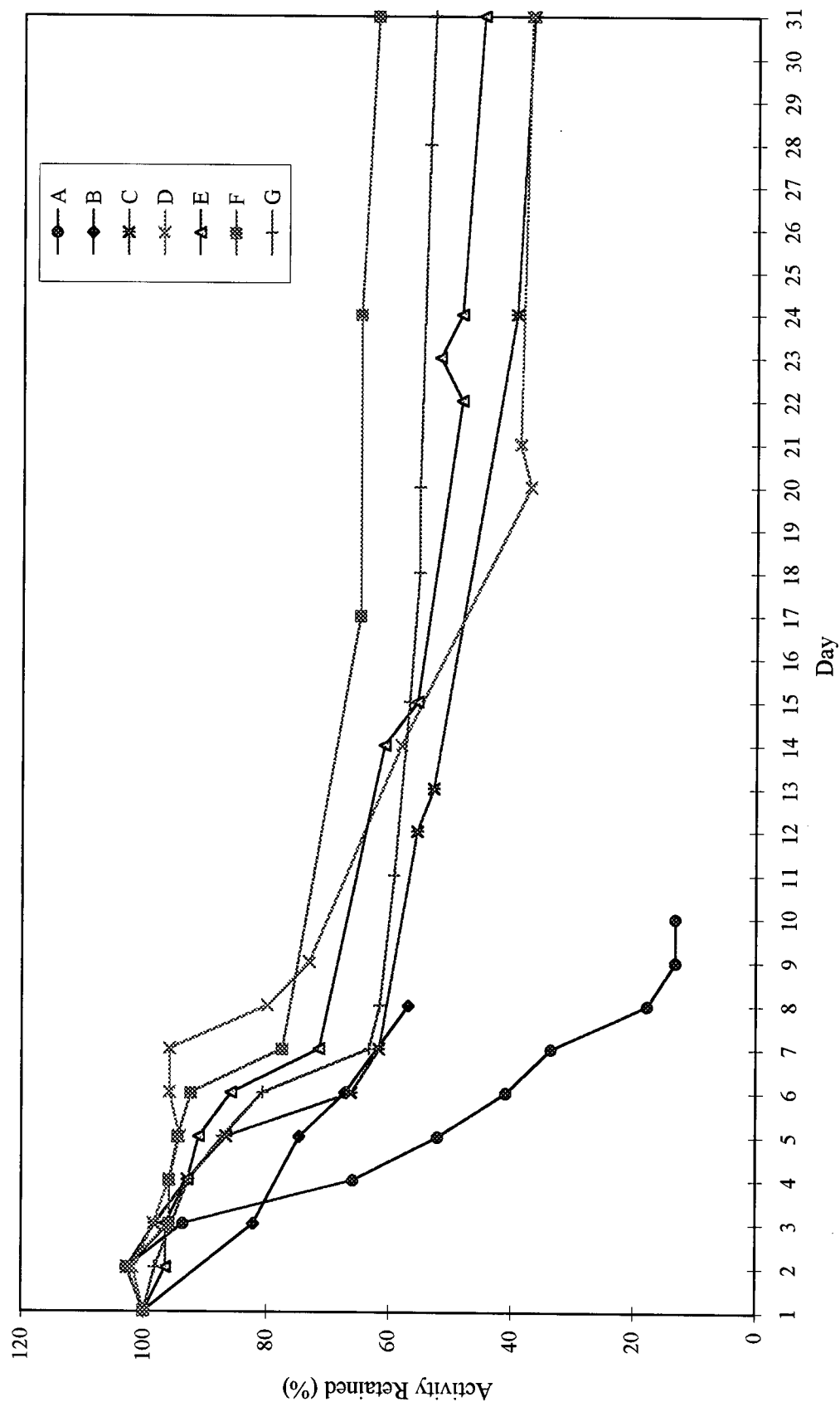
Figure 15. Stability of glucosidase I isolated with various combination of additives (Letters A - G in the legend refer to Combinations A - G used to stabilize glucosidase I during isolation and storage, as Combinations A - G in Table 3).

Moreover, the enzyme seemed to lose its activity very quickly during the first week of storage and to stabilize after this. For glucosidase I isolated with 10 mM phosphate buffer only, the enzyme lost most of its activity (around 15% left) in the first nine days of storage. However, since the data was only based on single isolation due to the limited amount of trisaccharide substrate, it was unable to conclude which combination was the best. Based on the data shown, the additives used during the isolation steps, the protease inhibitor cocktail, glycerol, PMSF, EDTA and BSA, could prolong the stability of the enzyme during storage. In practical terms, enzyme used for later studies in this research was isolated with Combination D (10 mM phosphate buffer, 10% glycerol, and 100 μM PMSF) due to the relatively good enzyme activity and stability obtained, its simplicity and low cost.

The possible effect of bacteria on the stability of glucosidase I during storage was tested by comparing the stability of sterile and non-sterile enzyme stored at 4°C. The result in Fig.16 showed that same trend in loss of enzyme activity occurred to both sterile and non-sterile samples throughout the one-month storage. Therefore, it was reasonable to conclude that proteolysis by bacterial contamination did not contribute to the decreased enzyme activity observed in this work under the experimental conditions used.

As for freeze drying and freezing treatments, it was shown in Fig.17 that the stability of enzymes that had undergone freeze drying and freezing treatments behaved very similarly to that of enzyme without any treatment (i.e. stored at 4°C after isolation). The enzyme activity was very unstable for the first week and then dropped down more slowly. Among all samples, the activity of enzymes being frozen at –25°C and –80°C decreased much more gradually over time than that of enzyme that was freeze dried and that of enzyme without treatment. However, freezing temperature did not seem to be an important factor for
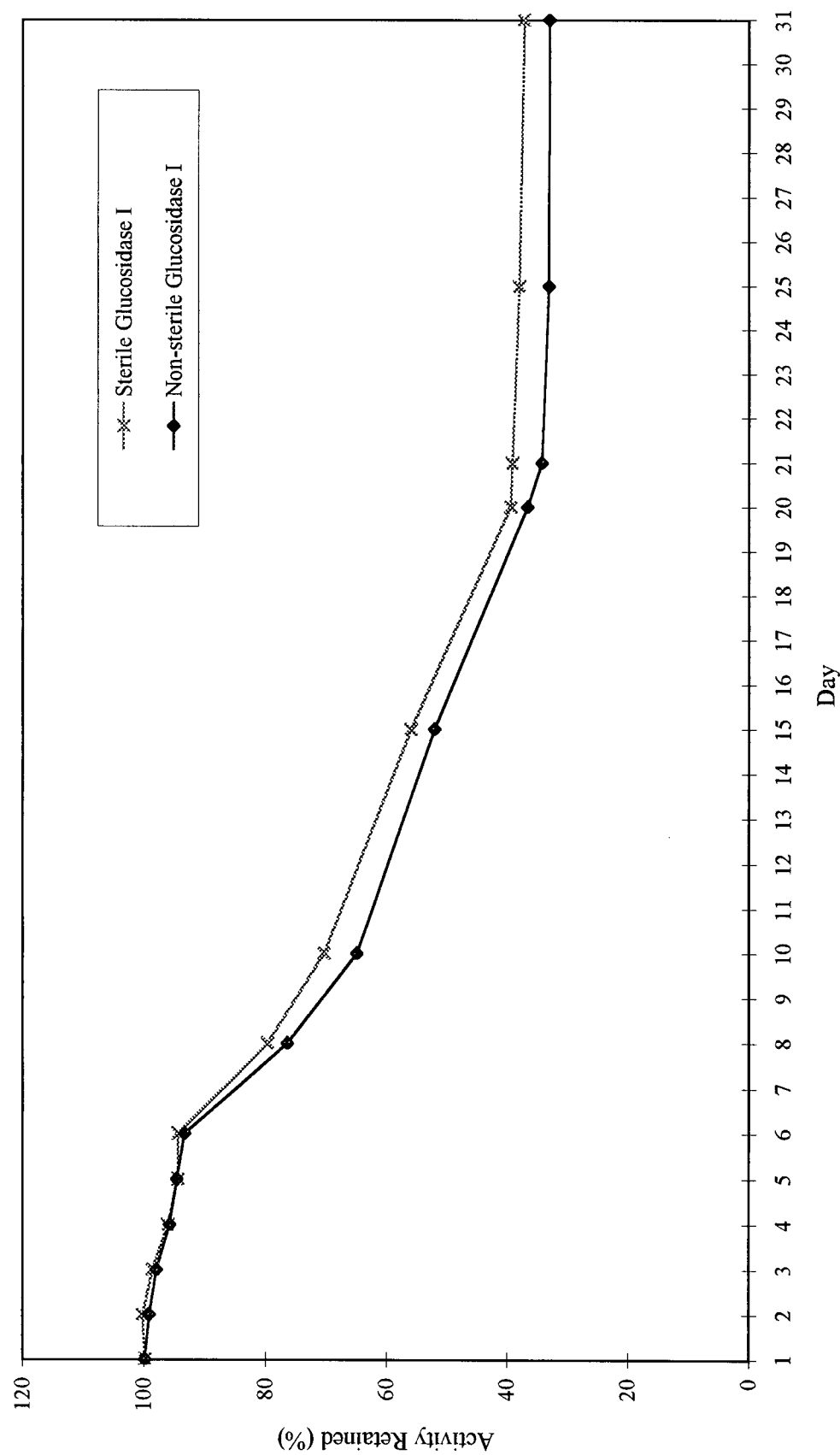
Figure 16. Stability of glucosidase I stored under sterile conditions compared to glucosidase I stored under non-sterile conditions. Enzyme was isolated with Combination D (10 mM phosphate buffer, 10% glycerol and 100 μM PMSF).
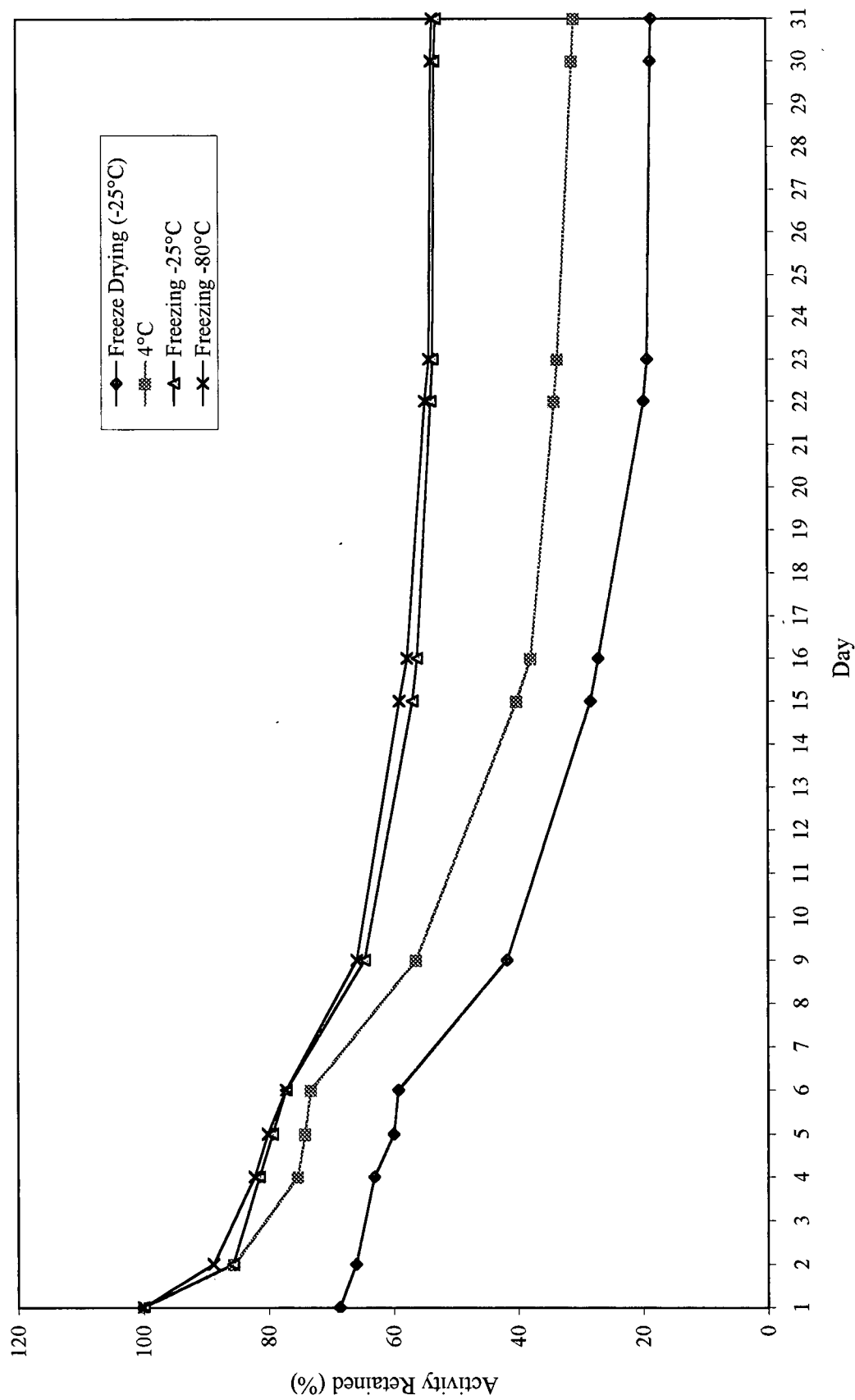
Figure 17. Stability of glucosidase I after freeze drying and freezing treatments.

enzyme stability, as the enzyme kept at –80°C lost activity at a very similar rate to enzyme kept at –25°C. In addition, about 35% of glucosidase I activity was lost after being freeze dried. The above results indicated that freezing could greatly increase glucosidase I stability during storage (had around 60% activity after a month, compared to having about 40% activity after 30 days when kept at 4°C, after isolation with the same additives - Combination D, refer to Fig.15) while freeze drying offered little help in terms of retention of activity.

4.3. Further Purification of Glucosidase I with Affinity Chromatography

4.3.1. Affinity Chromatography with N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin as the Ligand

Earlier studies demonstrated that 1-deoxynojirimycin (DNJM) is a good inhibitor for a variety of α- and β-glucosidases including glucosidase I and II (Fuhrmann *et al.*, 1985; Elbein, 1987). Recently it was noticed that the inhibitory effect of DNJM changed dramatically and in the opposite direction for the two enzymes when the ring nitrogen was alkylated. Thus glucosidase I was significantly more inhibited by the N-methyl derivative of DNJM than by DNJM itself, whereas the inhibition of glucosidase II was impaired several-fold by this modification (Bause *et al.*, 1989). Such different effects on glucosidase I and II formed the rationale for the use of N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin as the affinity ligand to further purify glucosidase I.

In this experiment, glucosidase I activity was found to be absent in the supernatant after being tumbled overnight with the packed gel. It indicated that glucosidase I was either successfully bound to the affinity matrix or had been inactivated during the tumbling. In addition, the purity of the isolated glucosidase I based on specific activity was improved by more than 468 times in terms of specific activity. However, the recovery of total enzyme activity was very low, with only about 0.41%. SDS-PAGE gel also showed that the affinity matrix was not very selective since several bands were observed on the gel. The failure of the experiment might be due to two reasons: 1) tumbling overnight might have denatured the enzyme; and 2) N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, the affinity ligand, was not successfully made.

4.3.2. Concanavalin A-Sepharose Chromatography

In contrast to the above inhibitor affinity chromatography, the use of Concanavalin A-Sepharose chromatography as an affinity resin for purification of glucosidase I was more promising. Although the purity of the isolated glucosidase I was improved by only 6.7 times based on specific activity, the recovery of total enzyme activity reached 32.7%. In addition, only two bands were observed on SDS-PAGE gel (with MW of 92.6 and 79.7 kD respectively) and one of the two bands calculated at 92.6 kD was quite close to the suggested molecular weight of 95 kD for the membrane bound form of glucosidase I (Fig.18). The molecular mass of soluble form of glucosidase I has not been determined so far. As well, it is not sure as yet if the soluble form of the enzyme is a result of a proteolytic cleavage of the membrane bound form or less likely – the soluble form is an independent gene product, which potentially might not be related to the membrane bound form.

Confirmation of the amino sequence of the 92.6 KD band purified from Con A-Sepharose chromatography was also attempted. The detailed procedure, including two steps – electrophoresis and electrotransfer, was as described in Section 3. Every effort was made to avoid interference with the sequencing reaction and to ensure efficient protein transfer. During the electrophoresis step, gel solutions except running buffer, were filtered, with a 0.45 micron filter; samples with solubilizing buffer were heated at 37°C for 10 to 15 minutes prior to loading onto the gel instead of being heated at 100°C for 1 minute; the cast gel was allowed to stand for 24 hours at room temperature prior to use to allow the gel, including stacker, to polymerize completely; 11.4 mg/l (0.1 mM) thioglycolate was added to the upper running buffer prior to electrophoresis to scavenge reactive compounds left in the gel which could cause N-terminal blocking; and acetic acid was avoided in staining (0.1%

200
116
97.4
66
45
31
21.5
14.5
6.5

Lane 1   Lane 2
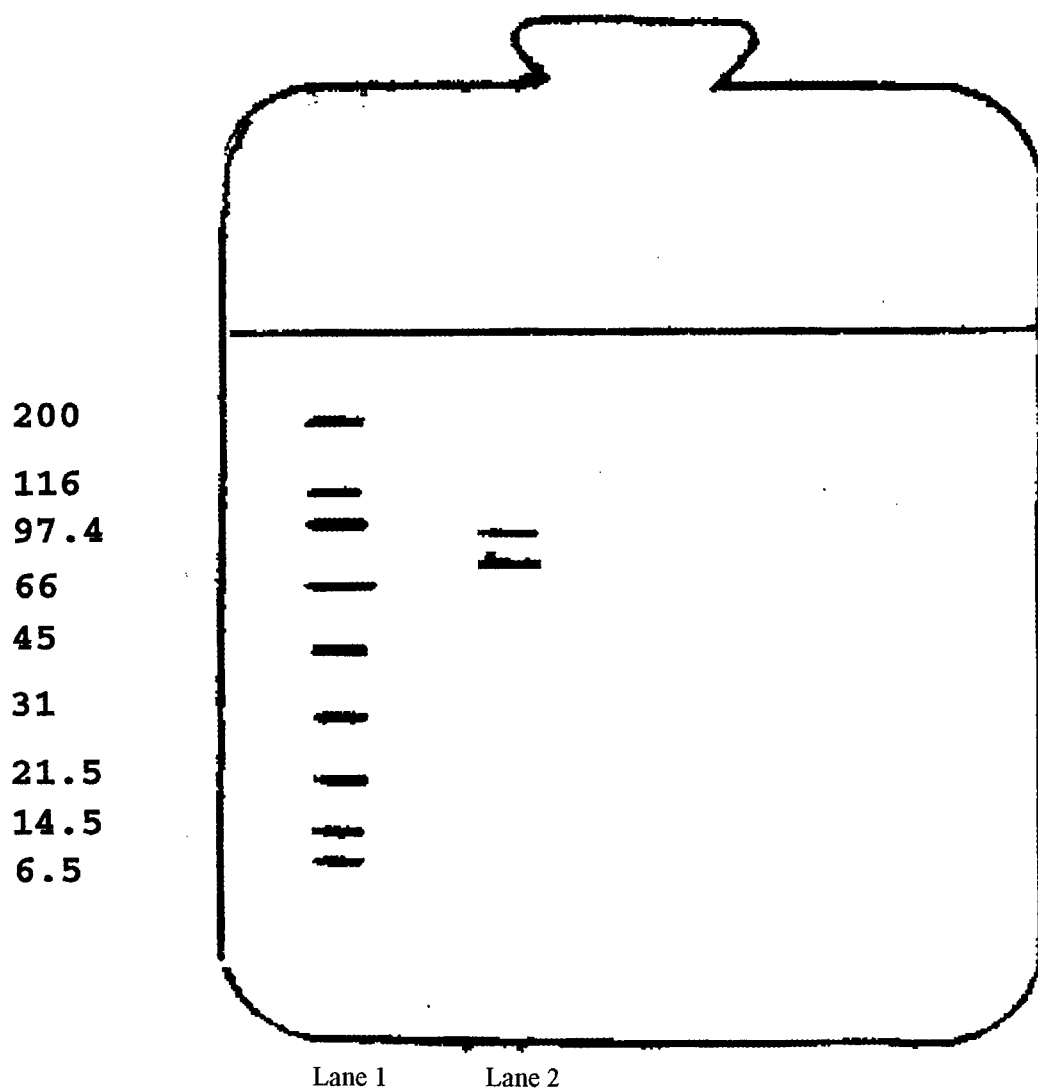
Figure 18. SDS-PAGE pattern of enzyme fraction from Con A-sepharose chromatography. Bands in Lane 1 represent the broad range SDS-PAGE standard, of which molecular weight of each band is showed on the left. Bands in Lane 2 represent the purified enzyme fraction.

Coomassie Blue in 50% methanol) and destaining (50% methanol) as it might cause

blockage of the amino terminus. During the electrotransfer step, PVDF membrane was

completely wetted with 100% methanol and equilibrated with CAPS transfer buffer [10mM

3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0] to ensure proper

binding; the high pH of the CAPS buffer assured transfer of most proteins; and CAPS buffer

contained no Tris or glycine, which would interfere with the sequencing reaction. However,

no clear result was obtained because of many interfering signals present during the amino

sequencing analysis. These interfering signals were most probably from glycine used at the

electrophoresis step. They could also come from other protein impurities mixed with the

purified glucosidase I. In addition, N-terminal blockage might have still occurred during the

sequencing. Reducing agents such as 2-mercaptoethanol or dithioerythritol were not added

to buffers used for the isolation and purification steps since they could interfere with the

glucosidase I activity assay. Lack of such reducing agents could lead to formation of

aggregates through disulfide bridge formation and the attack of the amino group by radicals,

aldehydes or oxygen, which in turn may have led to N-terminal blockage.

4.4. Stereochemistry of Glucosidase I Reaction

The stereochemical course of glucosidase I reaction can be determined using ^{1}H

NMR spectroscopy to directly monitor the release of glucose from the synthetic

trisaccharide substrate αGlc(1-2) αGlc(1-3)αGlc-O-(CH$_2$)$_8$COOCH$_3$. The time course of

hydrolysis catalyzed by the yeast glucosidase I is shown in Fig.19 where partial spectra of

the anomeric region are shown. The signals corresponding to the anomeric protons of each

of the glucose residues in the trisaccharide substrate, H1" at 5.54 ppm, H1' at 5.17 ppm and

H1 at 4.91 ppm and disaccharide product H1' at 5.34 ppm were well resolved from each

other and from free glucose. Three minutes after the addition of enzyme, the anomeric

proton of β-glucose was present at $\delta = 4.64$ ppm, as well as disaccharide product H1' signal

at 5.34 ppm. The β-glucose – H1 signal reached the 5% level after 13 minutes. This level

was reached by the α-anomer – H1 after 53 minutes, indicating a lag time of 40 minutes.

The yeast α-glucosidase I reaction therefore occurs with inversion of anomeric

configuration. After 5.5 hr the H1" trisaccharide as well as H1' trisaccharide signal

disappeared into the noise of the spectrum while the buildup of H1' disaccharide was

completed. In addition, the disaccharide product (after one glucose was cleaved from the

trisaccharide) was built up but no conversion to monosaccharide was seen. It indicates that

glucosidase II does not appear to be present in the isolated glucosidase I, or the amount of

residual glucosidase II does not seem to be enough to hydrolyze a significant amount of

disaccharide in the time period of the experiment or the disaccharide is a poor substrate.

Since the above results prove that glucosidase I is an inverting enzyme, and the

carboxylic side chains of aspartic or glutamic acids have been implicated in the catalytic

mechanism of other inverting glycosidases, it is likely that at least one acidic amino acid
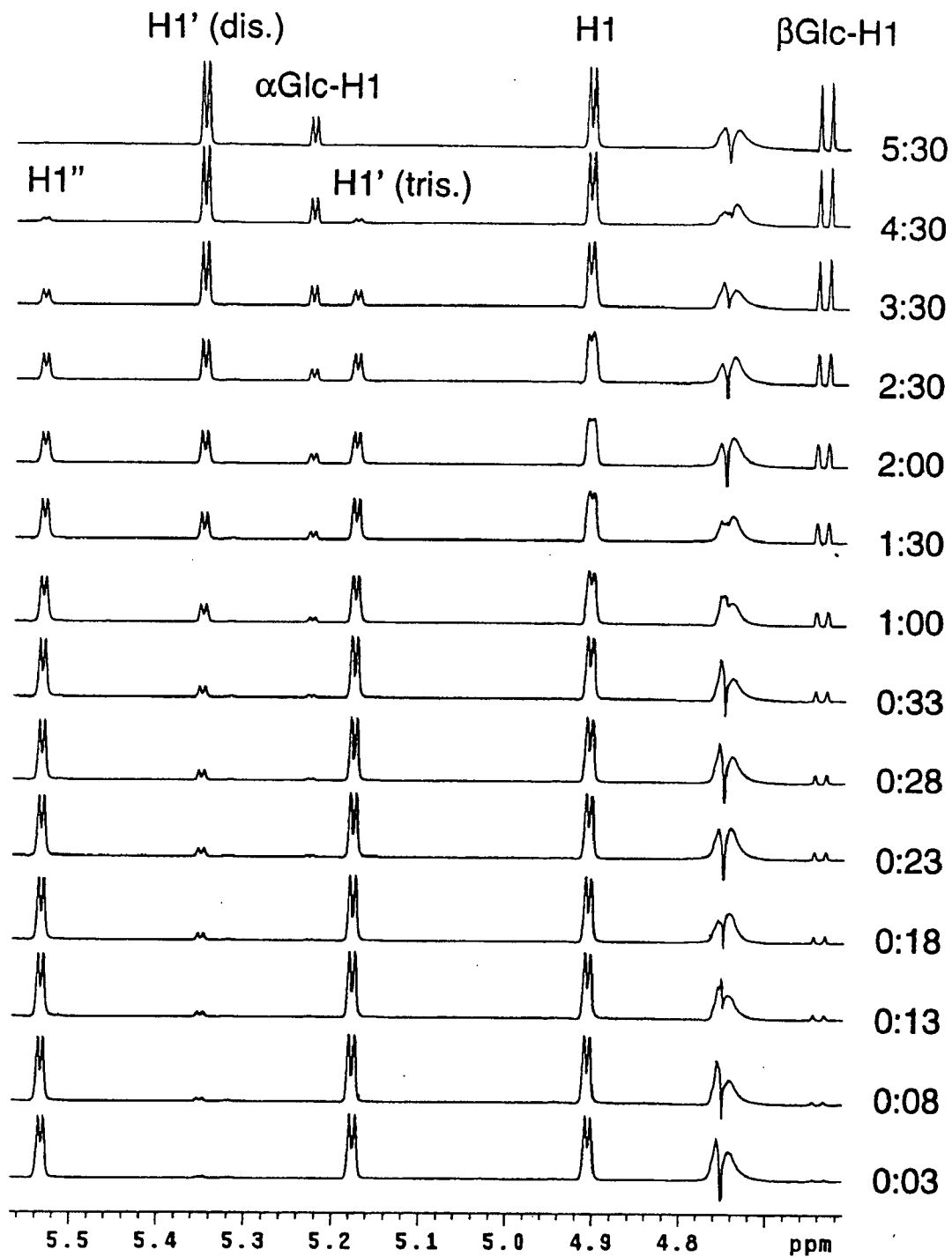
Figure 19. Time course of glucosidase I reaction (Palcic *et al.*, 1999).

which contains a carboxyl group is specifically involved in the active site of glucosidase I.

4.5. Kinetic Evaluation of Glucosidase I Substrate Analogues

Inhibitory effects of 11 synthetic substrate analogues were investigated by calculating the percentage enzyme activity remaining when these substrate analogues were added into the isolated enzyme. For substrate analogues that were found to have relatively potent inhibitory effect on the enzyme, the K_m (apparent) and V_{max} values were obtained using a direct fit of velocity and substrate data to the Michaelis-Menton equation, using nonlinear regression analysis. By re-plotting K_m (apparent) against substrate analogue concentration, K_i value of the substrate analogue was calculated as the negative x intercept of the re-plot, obtained by linear regression. Among the glucosidase I substrate analogues tested in this research, 2'Fluoro-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ and 2'N_3-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ showed the most potent inhibitory action against glucosidase I (decreased ~60% at 10 mM), while the others were less effective inhibitors (Table 7). There was no effect on the glucosidase I coupling assay components, when the analogues were present at 1 and 10 mM. Results of the kinetic evaluation of these two compounds are shown in Figs.20 and 21 respectively. It was indicated that V_{max} values calculated from reactions with different substrate analogue concentrations were approximately equal while K_m values increased with increasing substrate analogue concentrations. Therefore, the mechanism of inhibition is suggested to be competitive type, i.e. 2'Fluoro-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ and 2'N_3-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ are both competitive inhibitors of glucosidase I. In addition, a linear relationship between the apparent K_m and inhibitor concentration exists for these two compounds (Figs.22 and 23), from which the K_i values of 2'Fluoro-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ and 2'N_3-αGlc(1-2)αGlc-O-$(CH_2)_8COOCH_3$ were calculated to be 0.67 mM and 0.80 mM respectively. Due to their

Table 7. Inhibition of glucosidase I by substrate analogues at concentrations of 1 and 10 mM

Substrate analogues	% Glucosidase I activity remaining[a]	
	1 mM	10 mM
3'Deoxy-αGlc(1-2)αGlc-O-grease	92.05 ± 2.86	86.55 ± 1.42
4'Deoxy-αGlc(1-2)αGlc-O-grease	97.83 ± 1.22	96.28 ± 1.91
6'Deoxy-αGlc(1-2)αGlc-O-grease	77.60 ± 1.97	69.58 ± 2.23
O-MethylαGlc(1-2)αGlc-O-grease	96.27 ± 2.17	73.83 ± 1.73
2'O-MethylαGlc(1-2)αGlc-O-grease	79.04 ± 2.01	65.34 ± 2.54
4'O-MethylαGlc(1-2)αGlc-O-grease	95.57 ± 1.91	89.45 ± 1.52
2'NH$_2$-αGlc(1-2)αGlc-O-grease	89.86 ± 1.66	57.04 ± 1.57
2'N$_3$-αGlc(1-2)αGlc-O-grease	74.63 ± 1.27	42.46 ± 1.08
6'Chloro-αGlc(1-2)αGlc-O-grease	99.38 ± 0.82	71.63 ± 2.11
6 Chloro-αGlc(1-2)αGlc-O-grease	91.76 ± 1.36	50.56 ± 2.14
2'Fluoro-αGlc(1-2)αGlc-O-grease	75.58 ± 1.77	38.41 ± 1.62

[a] Data is based on duplicate samples.
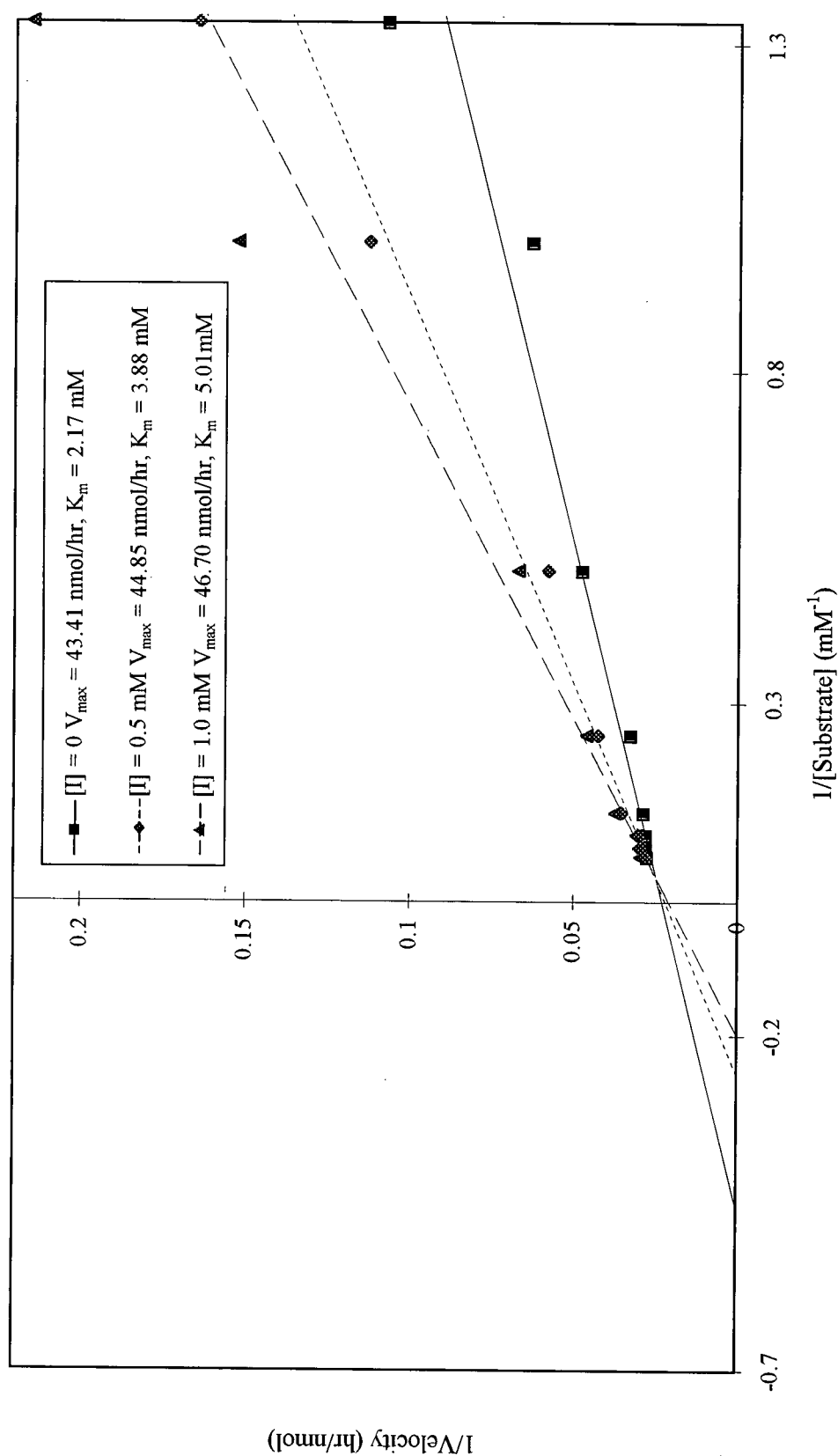
Figure 20. Lineweaver Burk inverse plot of $2'N_3$-αGlc$(1$-$2)\alpha$Glc-O-grease inhibition of glucosidase I. The original data was fit by non-linear regression to the Michaelis-Menton equation to obtain K_m (apparent) and V_{max} at different inhibitor concentations.

The figure legend indicates:

— [I] = 0 V_{max} = 43.41 nmol/hr, K_m = 2.17 mM

--- [I] = 0.5 mM V_{max} = 44.85 nmol/hr, K_m = 3.88 mM

--- [I] = 1.0 mM V_{max} = 46.70 nmol/hr, K_m = 5.01mM

x-axis: 1/[Substrate] (mM^{-1})

y-axis: 1/Velocity (hr/nmol)

70

Figure 21. Lineweaver Burk inverse plot of 2'Fluoro-αGlc(1-2)αGlc-O-grease inhibition of glucosidase I. The original data was fit by non-linear regression to the Michaelis-Menton equation to obtain K_m (apparent) and V_{max} at different inhibitor concentrations.

Legend (within plot):

— [I] = 0 V_{max} = 32.11 nmol/hr, K_m = 1.86 mM

--◈-- [I] = 1.0 mM V_{max} = 35.15 nmol/hr, K_m = 7.01 mM

--▲-- [I] = 2.5 mM V_{max} = 37.32 nmol/hr, K_m = 11.14 mM

X-axis: 1/[Substrate] (mM⁻¹)

Y-axis: 1/Velocity (hr/nmol)

71
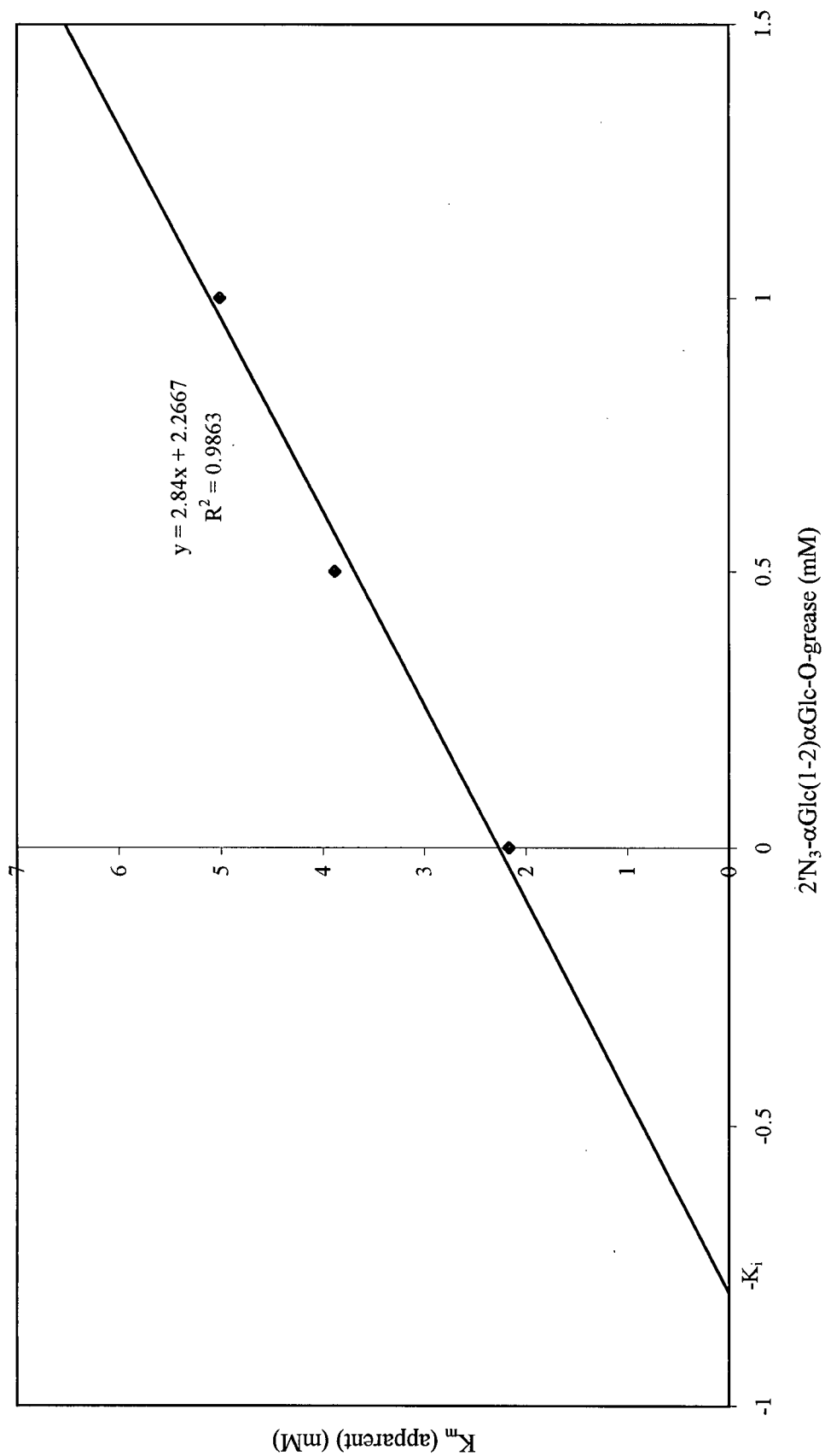
Figure 22. Replot of apparent K_m obtained from non-linear regression analysis of inhibition of glucosidase I at 0, 0.5 and 1.0 mM of $2'N_3-\alpha Glc(1-2)\alpha Glc$-O-grease (see Fig.20). K_i was calculated as the negative x-intercept.
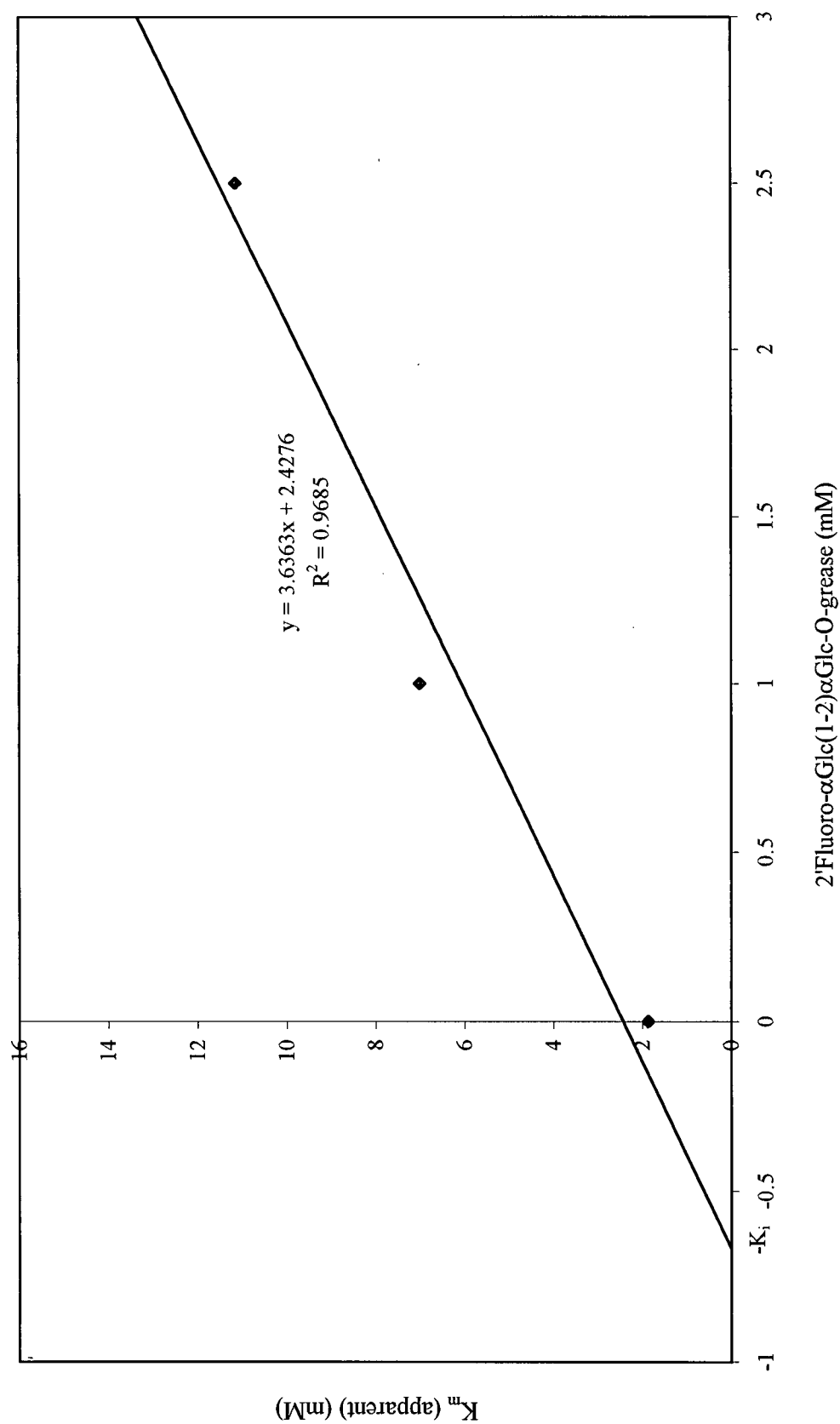
Figure 23. Replot of apparent K_m obtained from non-linear regression analysis of inhibition of glucosidase I at 0, 1.0 and 2.5 mM of 2'Fluoro-αGlc(1-2)αGlc-O-grease (see Fig.21). K_i was calculated as the negative x-intercept.

relatively less potent inhibitory effect on glucosidase I (50% inhibition achieved at about 10 mM), compared to deoxynojirimycin which inhibits the enzyme activity by 50% at 50 μM (at substrate concentration of 0.4 K_m) (Neverova *et al.*, 1994), the two compounds in the present study would not be useful as therapeutic agents.

4.6.   Site-Specific Chemical Modification of Glucosidase I

The effects of various concentrations of EDAC on glucosidase I activity are shown in Fig.24. Increasing glucosidase I inhibition was found as the concentration of EDAC increased from 10 to 50 mM, and inhibition also increased with incubation time, indicating that the primary structure of glucosidase I is composed of carboxyl group containing amino acids. In order to investigate further whether carboxyl group containing amino acids are present at the active site of the enzyme, EDAC at 50 mM which showed the most inhibitory action (about 90% inhibition when incubated for 60 min) in Fig.24 was selected for the next experimental procedure.

In the next experimental procedure, glucosidase I was treated with 10 mM DNJM and/or 50 mM EDAC. The results are shown in Figs.25 and 26. Glucosidase I activity decreased to about 8.5% of the original after addition of 10 mM DNJM. A slight decrease (less than 3%) in enzyme activity was observed when 50 mM EDAC was added into the enzyme and DNJM mixture. However, about 60% of the original enzyme activity were recovered after dialysis overnight during which DNJM and the excess EDAC were removed. When 50 mM EDAC was added to this recovered enzyme activity, it was again inhibited to about 10%. Therefore, it seemed that DNJM could protect the active site of the enzyme so that EDAC could not freely interact with the amino acid residues at the active site. The interaction between DNJM and the active site residues was reversible. On the other hand, dialysis overnight did not help to recover enzyme activity when the enzyme was treated with 50 mM EDAC only. Such modification was irreversible under the conditions used in this experiment.

EDAC is one of the water-soluble carbodiimides that have been widely used to
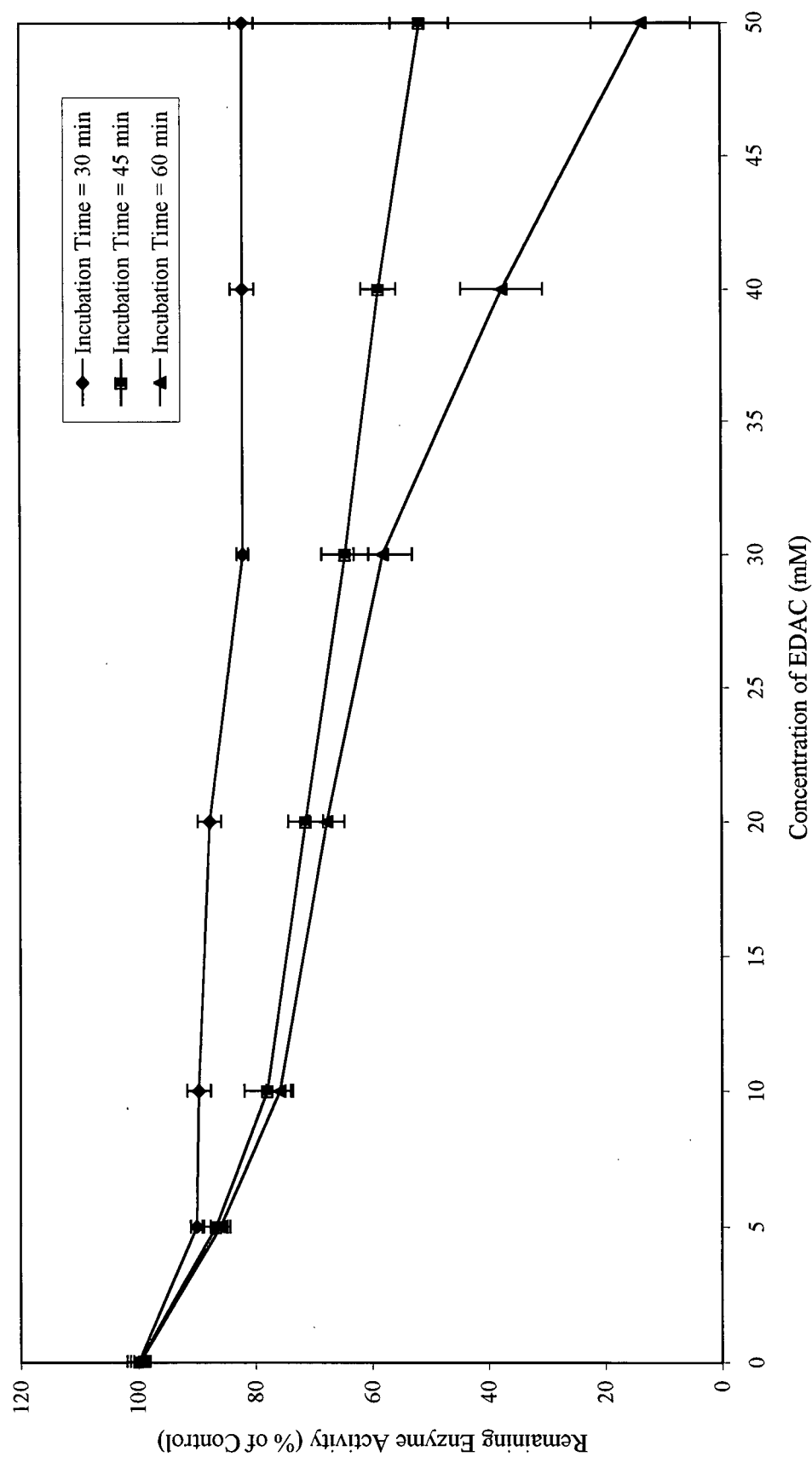
Figure 24. Inhibitory effect of EDAC on glucosidase I activity. Enzyme was isolated using buffers containing Combination D additives (10 mM phosphate buffer, 10% glycerol and 100 μM PMSF). Results presented are the average of duplicate assays at each time point. Error bars represent the range for each pair of readings.
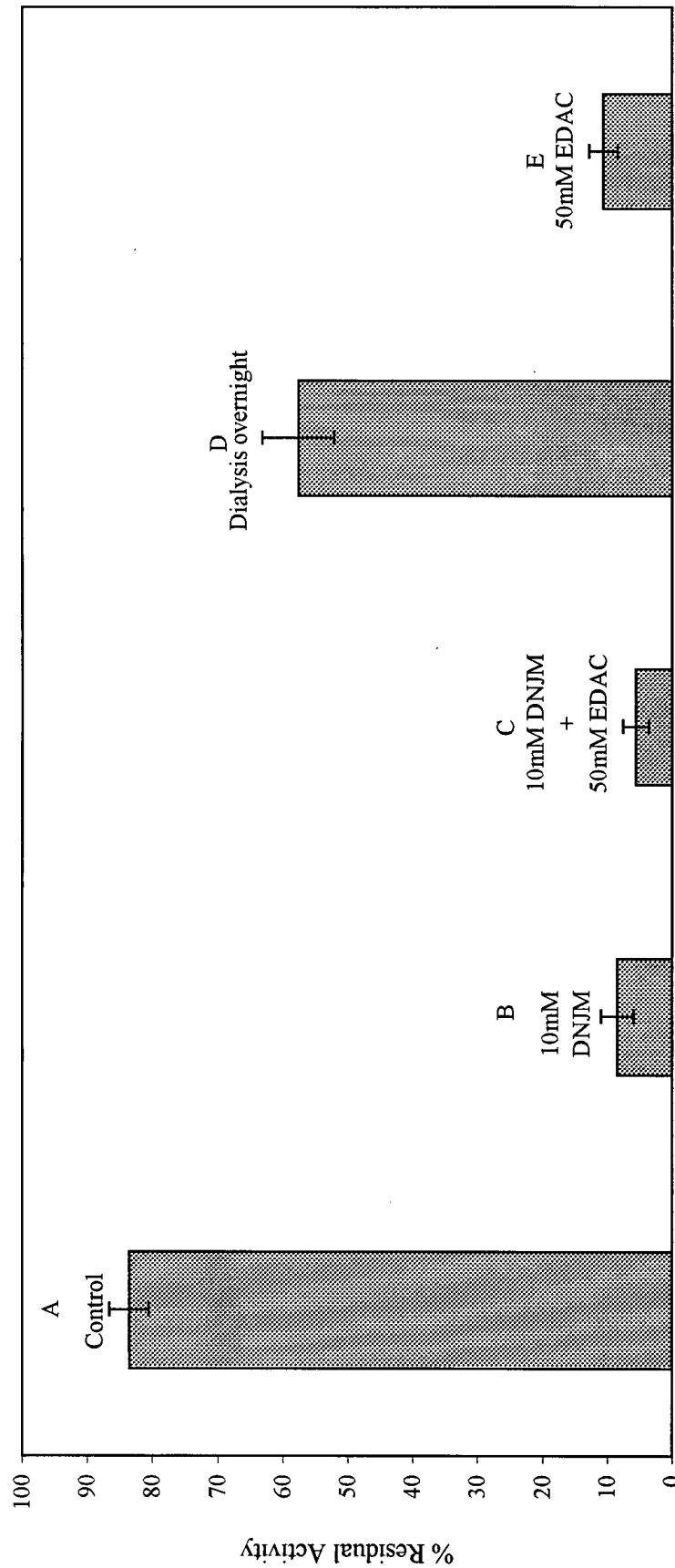
Figure 25. Percentage of initial enzyme activity remaining in samples of glucosidase I. Treatment A - dialysis in buffer containing10 mM phosphate, 10% glycerol and 100 μM PMSF, pH 6.8 (buffer A) overnight; Treatment B - added with 10 mM DNJM and incubated at 4°C for 1 hr; Treatment C - added with 50 mM EDAC and incubated at 4°C for 1 hr after Treatment B; Treatment D - dialysis in buffer A overnight after Treatment C; Treatment E - added with 50 mM EDAC and incubated at 4°C for 1 hr after Treatment D. Enzyme was isolated with Combination D additives (buffer A). Results presented are the average of duplicate assays at each time point. Error bars represent the range for each pair of readings.
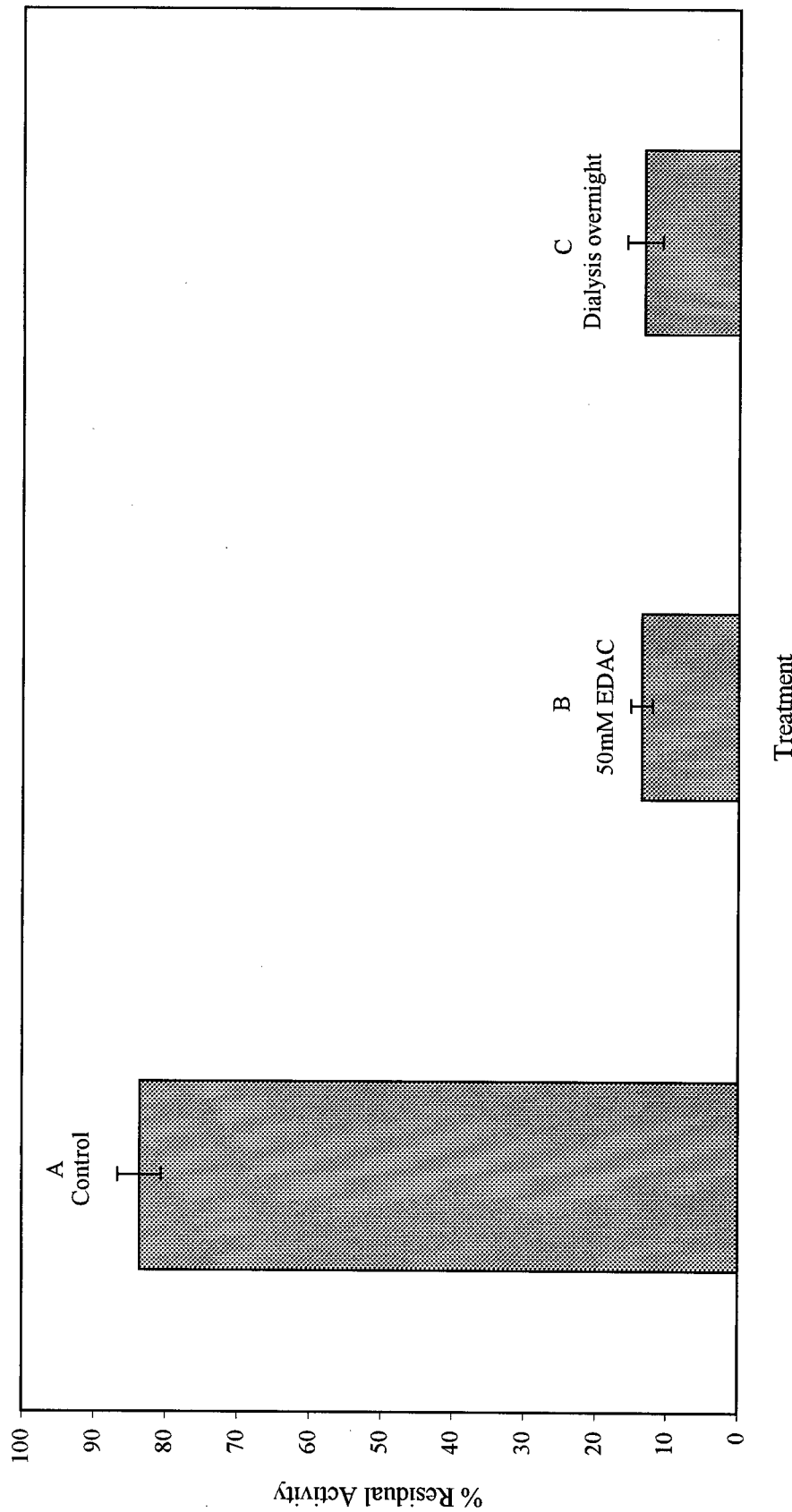
Figure 26. Percentage of initial enzyme activity remaining in samples of glucosidase I. Treatment A - dialysis in buffer containing 10 mM phosphate, 10% glycerol and 100 μM PMSF, pH 6.8 (buffer A) overnight; Treatment B - added with 50 mM EDAC and incubated at 4°C for 1 hr; Treatment C - dialysis in buffer A overnight after Treatment B. Enzyme was isolated with Combination D additives (buffer A). Results presented are the average of duplicate assays at each time point. Error bars represent the range for each pair of readings.

modify carboxyl groups in proteins. The reaction of carbodiimides with protein carboxyl groups can be seen in Fig.27 (Lundblad, 1995). The reaction sequence is initiated by addition of the carboxyl across one of the double bonds of the diimide system to give an O-acylisourea. The activated carboxyl group of this adduct can then react via one or two routes. First, an attack by a nucleophile will yield an acyl-nucleophile product plus the urea derived from the carbodiimide. Second, the O-acylisourea can rearrange to an N-acylisourea via an intramolecular acyl transfer (Khorana, 1953). Although modification of the carboxyl group in proteins with a water-soluble carbodiimide has proven to be extremely useful, there are problems with side reactions (Carraway and Koshland, 1972). Carbodiimides are not specific for carboxyl groups alone. Under the conditions employed in this investigation, EDAC would also react with aromatic hydroxyl groups of tyrosines and sulfhydryl groups of cysteines. Since the rate of reaction between tyrosine and EDAC is much slower than that between carboxyl groups and EDAC (Carraway and Koshland, 1968), and DNJM has been showed to be unable to protect glucosidase I from inactivation by tetranitromethane, a reagent specific for tyrosine (Romaniouk and Vijay, 1997), tyrosine is not likely to be present at the active site of glucosidase I. As for cysteines, Pukazhenthi *et al.* (1993) have suggested that a reactive cysteine was at or near the active site of glucosidase I. However, this does not deny the existence of a carboxyl group at the active site. It is also possible that at least a carboxyl group is involved in the inhibitory effect of EDAC on glucosidase I. There are examples of both cysteine and carboxyl groups being present at the active site of different glycosidases and the carboxyl amino acids playing the catalytic role have been found. Takayanagi *et al.* (1995) showed that both carboxyl and sulphydryl groups are present at the active site of beta-fructofuranosidase (E.C. 3.2.1.26) and both are essential

Protein–COOH + R–N=C=N–R' $\longrightarrow$

$$\text{Protein–}\overset{\overset{\textstyle O}{\|}}{C}\text{–O–}\overset{\overset{\textstyle HN-R'}{\|}}{\underset{\underset{\textstyle NH-R}{|}}{C}}$$

O-acylisourea

$$+H_2N\text{–}CH_2\overset{\overset{\textstyle O}{\|}}{C}\text{–}OCH_3$$

$$\text{Protein–}\overset{\overset{\textstyle O}{\|}}{C}\text{–}NH\text{–}CH_2\overset{\overset{\textstyle O}{\|}}{C}\text{–}OCH_3 \quad + \quad R\text{–}NH\text{–}\overset{\overset{\textstyle O}{\|}}{C}\text{–}NH\text{–}R'$$

$$\text{protein–}\overset{\overset{\textstyle O}{\|}}{C}\text{–}\overset{\overset{\textstyle O}{\overset{\|}{\textstyle C}}\text{–}NH\text{–}R'}{\underset{\underset{\textstyle R}{|}}{N}}$$
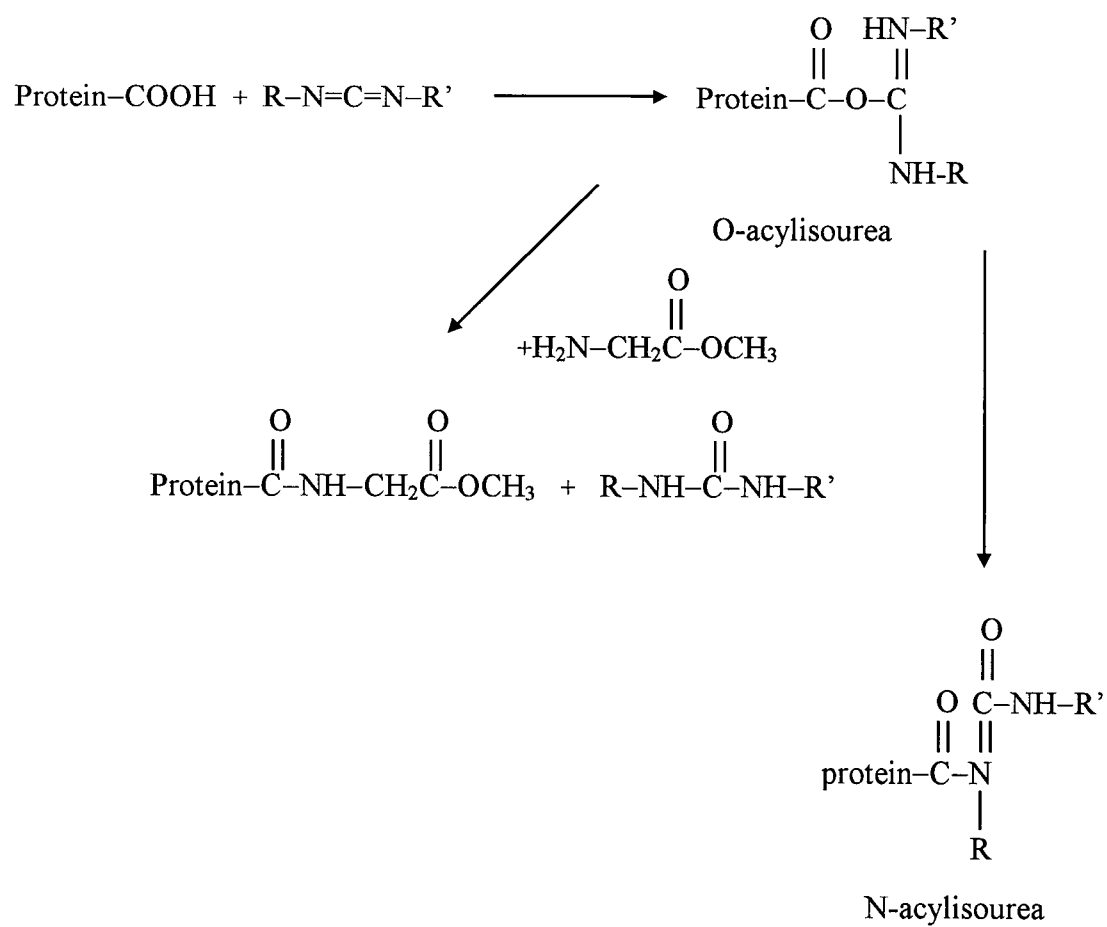
N-acylisourea

Figure 27. The reaction of carbodiimide with protein carboxyl groups.

for enzyme activity. Therefore, the result of modification of glucosidase I active site by EDAC in this research work either supports the presence of cysteine at the active site, or provides evidence that at least one acidic amino acid is located at the active site.

5. Conclusions

Glucosidase I is very unstable during isolation and storage under normal conditions (e.g. at 4°C). In this research, the stability of the enzyme was much improved by the addition of glycerol, EDTA, BSA, PMSF and protease inhibitors during isolation. When kept at extremely low temperature (-25°C and –80°C), the enzyme lost activity less rapidly than when stored at higher temperature (4°C). Freeze-drying treatment did not help to retain enzyme activity during storage.

Glucosidase I is shown to be an inverting enzyme which catalyzes hydrolysis with inversion of anomeric configuration and it can be further purified with Con-A chromatography. Kinetic study shows that substrate analogues 2'Fluoro-αGlc(1-2)αGlc-O-grease and 2'N$_3$-αGlc(1-2)αGlc-O-grease are competitive inhibitors of the enzyme. However, these two compounds would not be useful as glucosidase I inhibitors due to their relatively less potent inhibitory effect (only 50% inhibition at about 10 mM). In addition, modification of glucosidase I active site by EDAC either supports the presence of cysteine at the active site or provides evidence that at least an acidic amino acid is specifically involved in the active site of glucosidase I.

Although studies focused on the identification of the protein structure, the catalytically important active site residues, cloning and expression of the enzyme, and the role of the enzyme in the biosynthesis of cell wall components have begun, more work is needed in order to better understand the catalytic mechanism of glucosidase I and develop inhibitors as antiviral (HIV) and antitumor therapeutic agents.

6. References

Abeijon, C. & Chen, L.Y. (1998). The role of glucosidase I (Cwh41p) in the biosynthesis of cell wall β-1,6-glucan is indirect. *Mol. Biol. Cell* **9:** 2729 – 2738.

Aleshin, A.E., Firsov, L.M. & Honzatko, R.B. (1994). Refined structure for the complex of acarbose with glucoamylase from *Aspergillus awamori* var.X100 to 2.4 Å resolution. *J. Biol. Chem.* **269:** 15631 – 15639.

Bause, E., Erkens, R., Schweden, J. & Jaenicke, L. (1986). Purification and characterization of trimming glucosidse I from *Saccharomyces cerevisiae*. *FEBS Lett.* **206:** 208 - 212.

Bause, E., Gross, A. & Schweden, J. (1991). N-methyl-N-(5-carboxypentyl)-1-deoxynojirimycin, a new affinity ligand for the purification of trimming glucosidase I. *FEBS Lett.* **278:** 167 - 170.

Bause, E., Schweden, J., Gross, A. & Orthen, B. (1989). Purification and characterization of trimming glucosidase I from pig liver. *Eur. J. Biochem.* **183:** 661 – 669.

Bray, M.R. & Clarke, A.J. (1994). Identification of a glutamate residue at the active site of xylanase A from *Schizophyllum commune*. *Eur. J. Biochem.* **219:** 821 - 827.

Carraway, K.L. & Koshland, D.E., Jr. (1968). Reaction of tyrosine residues in proteins with carbodiimide reagents. *Biochim. Biophys. Acta.* **160:** 272 – 278.

Carraway, K.L. & Koshland, D.E., Jr. (1972). Carbodiimide modification of proteins. *Meth. Enzymol.* **25:** 616 – 622.

Chen, W.W. & Lennarz, W.J. (1978). Enzymatic excision of glucosyl units linked to the oligosaccharide chains of glycoproteins. *J. Biol. Chem.* **253:** 5780 - 5785.

Coughlan, M.P. & Hazlewood, G. (1993). Beta-1,4-D-xylan-degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnol. Appl. Biochem.* **17:** 259 - 289.

Dixon, M. & Webb, E.C. (1964). *Enzymes*, Longmans, Green and Co Ltd., London.

Elbein, A.D. (1987). Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* **56:** 497 – 534.

Elbein, A.D. (1991a). The role of N-linked oligosaccharides in glycoprotein function. *Trends Biotechnol.* **9:** 346 - 352.

Elbein, A.D. (1991b).Glycosidase inhibitors as antiviral and/or antitumor agents. *Sem. Cell Biol.* **2:** 309 - 317.

Elting, J.J., Chen, W.W. & Lennarz, W.J. (1980). Characterization of a glucosidase involved in an initial stop in the processing of oligosaccharide chains. *J. Biol. Chem.* **255:** 2325 - 2331.

Faye, L., Fitchette-Laine, A.-C., Gomord, V., Chekkafi, A., Delaunay, A.-M. and Driouich, A. (1993). Detection, biosynthesis and some functions of glycans N-linked to plant secreted proteins. In: *Post-Translational Modifications in Plants,* ed. N.H. Battey, H.G. Dickinson, and A.M. Hetherington, pp.213, Cambridge University Press, UK

Fenouillet, E., Gluckman, J.C. & Jones, I.M. (1994). Functions of HIV envelope glycans. *Trends Bio. Sci.* **19:** 65 - 70.

Fischer, P.B., Collin, M., Karlsson, G.B., *et al.* (1995). The alpha-glucosidase inhibitor N-butyldeoxynojirimycin inhibits human immunodeficiency virus entry at the level of post-CD4 binding. *J. Virol.* **69:** 5791 - 5797.

Frey, P.A. (1986). *Mechanisms of Enzymatic Reactions: Stereochemistry,* Elsevier Science Publishing Co., Inc., New York.

Fuhrmann, U., Bause, E. & Ploegh, H. (1985). Inhibitors of oligosaccharide processing. *Biochim. Biophys. Acta.* **825:** 95 – 110.

Grinna, L.S. & Robbins, P.W. (1979). Glycoprotein biosynthesis: rat liver microsomal

glucosidases which process oligosaccharides. *J. Biol. Chem.* **254:** 8814 - 8818.

Gruters, R.A., Neefjes, J.J., Tersmeette, M., de Goede, R.E., Tulp, A., Huisman, H.G., Miedema, F. & Ploegh, H.L. (1987). Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. *Nature* **330:** 74 - 77.

Hassing, G.S. & Goldstein, I.J. (1970). Ultraviolet difference spectral studies on concanavalin A carbohydrate interaction. *Eur. J. Biochem.* **16:** 549 - 556.

· Hettkamp, H., Legler, G. & Bause, E. (1984). Purification by affinity chromatography of glucosidase I, an endoplasmic reticulum hydrolase involved in the processing of asparagine-linked oligosaccharides. *Eur. J. Biochem.* **142:** 85 - 90.

Hibler, D.W., Stolowich, N.J., Mehdi, S. & Gerlt, J.A. (1986). Staphylococcal nuclease: stereochemical and genetic probes of the mechanism of the hydrolysis reaction. In: *Mechanisms of Enzymatic Reactions: Stereochemistry,* ed. P.A. Frey, pp.101, Elsevier Science Publishing Co., Inc., New York, NY.

Hubbard, S.C. & Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50:** 555-583.

James, T.L. (1975). *Nuclear Magnetic Resonance in Biochemistry,* Academic Press, New York, NY.

Kalz-Fuller, B., Bieberich, E. & Bause, E. (1995). Cloning and expression of glucosidase I from human hippocampus. *Eur. J. Biochem.* **231:** 344 – 351.

Khorana, H.G. (1953). The chemistry of carbodiimide. *Chem. Rev.* **53:** 145 – 154.

Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54:** 631 - 664.

Koshland, D.E., Jr. (1953). Stereochemistry and the mechanism of enzymatic reactions. *Biol. Rev.* **28:** 416 – 436.

Lundblad, R.L. (1995). The modification of carboxyl groups. In: *Techniques in Protein Modification,* pp.233, CRC Press, Boca Raton, FL.

Mikami, B., Hehre, E.J., Sato, M., Katsube, Y., Hirose, M., Morita, Y. & Sacchettini, J.C. (1993). The 2.0Å resolution structure of soybean beta-amylase complexed with alpha-cyclodextrin. *Biochemistry* **32:** 6836 – 6845.

Montefiori, D.C., Robinson, W.E., Jr. & Mitchell, W.M. (1988). Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **85:** 9248 - 9252.

Neverova, I., Scaman, C.H., Srivastava, O.P., Szweda, R., Vijay, I.K. & Palcic M.M.

(1994). A spectrophotometric assay for glucosidase I. *Anal. Biochem.* **222:** 190 - 195.

Niwa, T., Inouye, S., Tsuruoka, T., Koaze, Y. & Niida, T. (1970). Nojirimycin as a potent inhibitor of glucosidase. *Agric. Biol. Chem.* **34:** 966 - 968.

Palcic, M.M., Scaman, C.H., Otter, A., Szpacenko, A., Romaniuk, A., Li, Y.X. & Vijay, I.K. (1999). Processing α-glucosidase I is an inverting glycosidase. *Glycobiology* Accepted.

Porile, N.T. (1987). *Modern University Chemistry,* Harcourt Brace Jovanovich, Publishers, San Diego, CA.

Pukazhenthi, B.S., Muniappa, N. & Vijay, I.K. (1993). Role of sulfhydryl groups in the function of glucosidase I from mammary gland. *J. Biol. Chem.* **268:** 6445 - 6452.

Reddy, A. & Maley, F. (1996). Studies on identifying the catalytic role of glu-204 in the active site of yeast invertase. *J. Biol. Chem.* **271:** 13953 – 13958.

Romaniouk, A. & Vijay, I.K. (1997). Structure-function relationships in glucosidase I: amino acids involved in binding the substrate to the enzyme. *Glycobiology* **7:** 399 - 404.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C. & Jones, T.A. (1990). Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei. Science* **249:** 380 – 386.

Sadhu, A. & Magnuson, A.M. (1989). Role of second metal ion in establishing active conformations of concanavalin A. *Biochemistry* **28:** 3197 - 3204.

Scopes, R. (1982). *Protein Purification: Principles and Practice,* Springer-Verlag, New York, NY.

Segel, I.H. (1975). *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems,* John Wiley & Sons, New York.

Shahinian, S., Dijkgraaf, G.J.P., Sdicu, A.-M., Thomas, D.Y., Jakob, C.A., Aebi, M. & Bussey, H. (1998). Involvement of protein N-glucosyl chain glucosylation and processing in the biosynthesis of cell wall β-1,6-glucan of *Saccharomyces cerevisiae. Genetics* **149:** 843 – 856.

Shailubhai, K., Pratta, M.A. & Vijay, I.K. (1987). Purification and characterization of glucosidase I involved in N-linked glycoprotein processing in bovine mammary gland. *Biochem. J.* **247:** 555 - 562.

Shailubhai, K., Pukazhenthi, B.S., Saxena, E.S., Varma, G.M. & Vijay, I.K. (1991). Glucosidase I, a transmembrane endoplasmic reticular glycoprotein with a luminal catalytic domain. *J. Biol. Chem.* **266:** 16587 - 16593.

Spezio, M., Wilson, D.B. & Karplus, P.A. (1993). Crystal structure of the catalytic domain

of a thermophilic endocellulase. *Biochemistry* **32:** 9906 – 9916.

Sumner, J.B. (1926). The isolation and crystallization of the enzyme urease. *J. Biol. Chem.* **69:** 435 – 445.

Sun, Z., Duke, S.H. & Henson, C.A. (1995). The role of pea chloroplast α-glucosidase in transitory starch degradation. *Plant Physiol.* **108:** 211 - 217.

Szumilo, T., Kaushal, G.P. & Elbein, A.D. (1986). Purification and properties of glucosidase I from mung bean seedlings. *Arch. Biochem. Biophy.* **247:** 261 - 271.

Takayanagi, T., Porntaveewat, W. & Yokotsuka, K. (1995). Estimation of active site residues essential for activity of grape invertase by chemical modifications. *J. Appl. Glycosci.* **42:** 121 – 127.

Thoma, J.A. & Koshland, D.E. (1960). Stereochemistry of enzyme, substrate, and products during β-amylase action. *J. Biol. Chem.* **235:** 2511 - 2518.

Tomme, P. & Claeyssens, M. (1989). Identification of a functionally important carboxyl group in cellobiohydrolase I from *Trichoderma reesei. FEBS Lett.* **243:** 239 - 243.

Ugalde, A., Staneloni, R.J. & Leloir, L.F. (1980). Microsomal glucosidases of rat liver. Partial purification and inhibition by disaccharide. *Eur. J. Biochem.* **113:** 97 - 103.

van den Broek, L.A.G.M., Kat-Van den Nieuwenhor, M.W.P., Butters, T.D. & van

Boeckel, C.A.A. (1996). Synthesis of α-glucosidase I inhibitors showing antiviral (HIV-1)

and immunosuppressive activity. *J. Pharm. Pharmacol.* **48:** 172 - 178.

Walker, B.D., Kowalske, M., Goh, W.C., Kozarsky, K., Krieger, M., Rosen, C.,

Rohrschneider, L., Haseltine, W.A. & Sodorski, J. (1988). Inhibition of human

immunodeficiency virus syncytium formation and virus replication by castanospermine.

Proc. Natl. Acad. Sci. USA **84:** 8120 - 8214.

Wiseman, A. & Gould, B.J. (1971). *Enzymes, Their Nature and Role*, Hutchinson

Educational Ltd., London.

Withers, S.G. & Aebersold, R. (1995). Approaches to labeling and identification of active

site residues in glycosidases. *Prot. Sci.* **4:** 361 – 372.

Appendix 1: Results of isolating glucosidase I with various combinations of additives including volume, protein content and enzyme activity of each fraction during isolation steps.

Table A. G-I isolated with Combination A (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.92	23.53	0.816	0.75	0.035
0.3M NaCl elution	1.80	1.14	0.698	1.26	0.612
Dialyzed enzyme protein (just before FPLC)	1.84	1.00	0.649	1.19	0.649
G-I collected	0.12	0.77	1.061	0.13	1.378

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Table B. G-I isolated with Combination B (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.60	37.87	0.460	0.28	0.012
0.3M NaCl elution	1.72	---	0.578	0.99	---
Dialyzed enzyme protein (just before FPLC)	1.84	---	0.512	0.94	---
G-I collected	0.40	0.80	0.586	0.23	0.733

Table C. G-I isolated with Combination C (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.88	28.89	0.580	0.51	0.020
0.3M NaCl elution	1.88	1.37	0.512	0.96	0.374
Dialyzed enzyme protein (just before FPLC)	1.88	1.44	0.492	0.93	0.342
G-I collected	0.20	0.95	1.100	0.22	1.158

Table D. G-I isolated with Combination D (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.92	22.69	0.531	0.49	0.023
0.3M NaCl elution	2.16	1.34	0.502	1.08	0.375
Dialyzed enzyme protein (just before FPLC)	2.16	1.68	0.433	0.94	0.258
G-I collected	0.40	0.91	1.169	0.47	1.284

Table E. G-I isolated with Combination E (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.88	37.43	0.482	0.42	0.013
0.3M NaCl elution	2.32	1.62	0.394	0.91	0.243
Dialyzed enzyme protein (just before FPLC)	2.32	1.61	0.384	0.89	0.239
G-I collected	0.44	0.82	1.102	0.48	1.344

Table F. G-I isolated with Combination F (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.94	42.68	0.610	0.57	0.014
0.3M NaCl elution	2.00	1.62	0.482	0.96	0.298
Dialyzed enzyme protein (just before FPLC)	2.02	1.76	0.460	0.93	0.261
G-I collected	0.52	2.23	1.396	0.73	0.626

Table G. G-I isolated with Combination G (per g yeast cells).

Fraction	Volume (ml)	Protein concentration (mg/ml)	Activity (nmol/μl of enzyme*hr)	Total activity (μmol/hr)	Specific activity (nmol/μg*hr)
Dialyzed crude protein (just before DEAE)	0.88	35.61	0.306	0.27	0.009
0.3M NaCl elution	2.64	0.98	0.375	0.99	0.382
Dialyzed enzyme protein (just before FPLC)	2.68	1.23	0.335	0.90	0.273
G-I collected	0.44	1.91	1.227	0.54	0.643