PURIFICATION AND CHARACTERIZATION OF THE SOLUBLE FORM OF PROCESSING α-GLUCOSIDASE I FROM SACCHAROMYCES CEREVISIAE

by

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Saccharomyces cerevisiae processing α-glucosidase I (Cwh41p) is a type II membrane-bound glycoprotein encoded by CWH41. This enzyme regulates the first trimming step in the N-glycosylation pathway and may play an important role in glycoprotein biosynthesis and quality control in the endoplasmic reticulum (ER). Despite its importance, there is a limited understanding of the structure, functional residues, and mechanism of α-glucosidase I. Therefore this thesis was focused on establishing a robust purification method for the soluble form of yeast α-glucosidase I, identifying the catalytic domain, and determining structural or functional amino acid residues.

The soluble form of α-glucosidase I was purified to 95% homogeneity using a combination of ammonium sulfate precipitation, anion-exchange, lectin affinity, and size-exclusion chromatographies. The molecular mass of soluble α-glucosidase I was 98 kDa by SDS-PAGE. The purification method was improved by cultivation of transformed yeast in a fermenter and using a deoxynojirimycin (DNM)-based column. This method reproducibly yielded 40 µg of pure enzyme per gram of wet biomass with no detectable contamination by other aryl α- and β-glucosidases. Cleavage between Ala24 and Thr25 of the transmembrane domain of Cwh41p released the soluble activity and this fragment was shown to be glycosylated.

A luminal 37 kDa non-glycosylated polypeptide was isolated as the smallest active fragment from endogenous and trypsin hydrolysis of the soluble α-glucosidase I, using DNM-based resins. The hydrolysis sites were determined to be between Arg521 and Thr522 for endogenous proteolysis and Lys524 and Phe525 for the trypsin hydrolysis. This 37 kDa polypeptide was 1.9 times more active than the 98 kDa protein when assayed with the synthetic trisaccharide.
Site-specific chemical modification of the soluble \( \alpha \)-glucosidase I from yeast using diethylpyrocarbonate, tetrani tromethane and 3-(3-(dimethylamino)propyl)carbodiimide revealed that histidine, tyrosine and carboxylic acid residues are involved in \( \alpha \)-glucosidase I activity, as these residues could be protected from modification using the competitive inhibitor DNM. DNM could not prevent inactivation of enzyme treated with N-bromosuccinimide used to modify tryptophan residues.

Functional expression of truncated forms of Cwh41p was also investigated. Only Cwht1p (E35- F833) was expressed as a catalytically active soluble fragment. Cwht1p was isolated as a 94 kDa non-glycosylated polypeptide with a specific activity (3600 U/mg of protein) comparable to the soluble \( \alpha \)-glucosidase I (3000 U/mg of protein). These results suggest that the M1- I28 region, containing the predicted N-terminal cytosolic segment and transmembrane domain, of Cwh41p likely carries an ER-targeting signal sequence and is not important for protein folding.

Alignment of orthologs indicated that six highly conserved carboxylic acid residues resided within the putative catalytic region of yeast \( \alpha \)-glucosidase I. Substitution with Ala for E580 and D584 of Cwht1p (E613 and D617 of Cwh41p), that are situated at the corresponding proposed binding motif of the mammalian enzyme, resulted in undetectable \( \alpha \)-glucosidase I activity. Furthermore, mutants were expressed at considerably lower concentrations than Cwht1p. These findings suggest that conserved E613 and D617 may play an important functional or structural role in enzyme activity.

In conclusion, results of this thesis indicated that the soluble \( \alpha \)-glucosidase I is a proteolytic product of Cwh41p and can be functionally expressed without undergoing \( N \)-glycosylation. Moreover, the catalytically active fragment (F525-F833) can be isolated from the soluble \( \alpha \)-glucosidase I but it can not be expressed alone suggesting an integral structural
function for the non-catalytic region (E53-F525). Also, chemical modification results suggest that the potential binding residues are more conserved between yeast and plant, rather than yeast and mammalian α-glucosidase I. Finally, the conserved E613 and D617 may play an important role in yeast α-glucosidase I activity.
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<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>alg</td>
<td>Asparagine-linked glycosylation defective mutant</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance unit</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Brij 58</td>
<td>Polyethylene glycol hexadecyl ether</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(Cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CDG</td>
<td>Congenital disorder of glycosylation</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CP-DNM</td>
<td>N-5’-Carboxypentyl-deoxynojirimycin</td>
</tr>
<tr>
<td>cwh</td>
<td>Calcofluor white hypersensitivity mutants</td>
</tr>
<tr>
<td>CWH41</td>
<td>Gene that encodes yeast processing α-glucosidase I</td>
</tr>
<tr>
<td>CWHT</td>
<td>Truncated form of CWH41</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>der</td>
<td>Degradation in the endoplasmic reticulum mutants</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNM</td>
<td>1-Deoxynojirimycin</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
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<td>Dol-P</td>
<td>Dolichyl-phosphate</td>
</tr>
<tr>
<td>Dol-P-P</td>
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</tr>
<tr>
<td>DTT</td>
<td>1,4-Dithio-DL-threitol</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme commission</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDAC</td>
<td>3-(3-(dimethylamino)propyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation of protein</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational acceleration constant (9.8 m/s²)</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>GT</td>
<td>UDP-Glc: glycoprotein glucosyltransferase</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>His-Trap HP</td>
<td>Nickel-Sepharose high performance column</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>IUBMB</td>
<td>International Union of Biochemistry and Molecular Biology</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases (1000 bp)</td>
</tr>
<tr>
<td>kcat</td>
<td>Catalytic rate constant</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>Specificity constant</td>
</tr>
<tr>
<td>Ki</td>
<td>Enzyme-inhibitor complex dissociation constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>Methyl-CP-DNM</td>
<td>N-Methyl-N-5′-carboxypentyl-deoxynojirimycin</td>
</tr>
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<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NB-DNM</td>
<td>N-Butyl-deoxynojirimycin</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PAGE</td>
<td>Polyacryl amide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>Phenyl glyoxal</td>
</tr>
<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase gene</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control of glycoproteins in the endoplasmic reticulum</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S</td>
<td>Seconds</td>
</tr>
<tr>
<td>TNM</td>
<td>Tetranitromethane</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-(hydoxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>U</td>
<td>Units of activity</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
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**Common Amino Acid Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Amino Acid</th>
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<tr>
<td>A</td>
<td>Ala</td>
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<tr>
<td>C</td>
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<td>D</td>
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<td>K</td>
<td>Lys</td>
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<td>L</td>
<td>Leu</td>
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</tr>
<tr>
<td>W</td>
<td>Trp</td>
</tr>
<tr>
<td>Y</td>
<td>Tyr</td>
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### Notes
- **SDS**: Sodium dodecyl sulfate
- **S**: Seconds
- **TNM**: Tetranitromethane
- **Tris**: 2-Amino-2-(hydoxymethyl)-1,3-propanediol
- **U**: Units of activity
- **UDP**: Uridine diphosphate
- **UV**: Ultraviolet
- **V**: Volts

### Amino Acid Abbreviations
- **A**: Alanine
- **C**: Cysteine
- **D**: Aspartic acid
- **E**: Glutamic acid
- **F**: Phenylalanine
- **G**: Glycine
- **H**: Histidine
- **I**: Isoleucine
- **K**: Lysine
- **L**: Leucine
- **M**: Methionine
- **N**: Asparagine
- **P**: Proline
- **Q**: Glutamine
- **R**: Arginine
- **S**: Serine
- **T**: Threonine
- **V**: Valine
- **W**: Tryptophan
- **Y**: Tyrosine
### Nucleotide Base Abbreviations

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<td>A</td>
<td>Adenine</td>
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<tr>
<td>C</td>
<td>Cytosine</td>
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<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
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PREFACE

Portions of this thesis have been published or accepted for publication and are listed below. The principal author of these papers is Amirreza Faridmoayer and the co-author is Dr. Christine H. Scaman (supervisor).


* Authors contributed equally: AF was responsible for establishing purification method and enzyme characterization (except enzyme modification with 3-(3-(dimethylamino)propyl)carbodiimide).
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In memory of Dr. Charles E. Warren (1962-2005)
CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW
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1.1 GENERAL INTRODUCTION

Glycosylation is the most common form of covalent modification of proteins, with more than 50% of proteins predicted to undergo glycosylation (Apweiler et al. 1999). Glycosylation is also considered to be the most complex co- and post-translational modification. It differs from other covalent modifications, such as phosphorylation, acetylation, formylation and biotinylation with respect to the size and the diversity of the added groups (glycans) and the magnitude of the cellular machinery devoted to synthesis and modulation (Charlwood et al. 2001). Thirteen different monosaccharides and eight amino acids participate in glycosylation to make at least 41 different sugar-amino acid combinations (Spiro 2002). To encompass such diverse modification, the glycosylation machinery employs numerous glycosidases and is controlled by factors that vary greatly among cell types and species (Spiro 2002). The attachment of glycans to a protein alters characteristics such as solubility, charge, structural rigidity, and conformation (Helenius & Aebi 2004). Due to the structural complexity and diversity of the linked glycans it is not plausible to suggest a single functional role for them. However, the common function proposed for the linked glycans is to provide additional recognition epitopes for proteins which are used in bio-recognition processes (Gabius et al. 2004).

Glycoproteins are categorized into different classes based on the glycosidic linkages between sugar and protein. The main groups are N-linked (or asparagine-linked), O-linked glycans, and glycosylphosphatidyl inositol (GPI) anchors (Rudd & Dwek 1997). Analyzing well-characterized glycoproteins from the Swiss-Prot database has revealed that approximately 90% carry N-glycosidic bonds. Therefore it was speculated that N-glycosylation is the predominant form of glycosylation (Apweiler et al. 1999).
In eukaryotic cells N-glycosylation of proteins is initiated in the endoplasmic reticulum (ER) and is completed in the Golgi. The early stage of N-glycosylation includes transfer of the oligosaccharide from its lipid carrier to the Asn residue of the nascent sequence of the polypeptide and subsequent processing of the N-glycan which takes place in the ER (Kornfeld & Kornfeld 1985). Processing of the newly assembled N-glycans is accomplished with a series of ER glycosidases, such as α-glucosidases (I and II) and α-mannosidase(s) which remove glucose and mannose residues, respectively (Herscovics 1999b). These processing steps introduce limited diversity to the glycan core and the structure of the N-glycan remains rather uniform until the glycoproteins reach the Golgi, where additional modifications take place (Helenius & Aebi 2001).

Processing α-glucosidase I commences trimming of the newly assembled N-glycans by removing the outermost glucose residue from the oligosaccharide core (Chen & Lennarz 1978; Grinna & Robbins 1979). This enzyme has been found in virtually all eukaryotes (Grinna & Robbins 1979; Kilker, Jr. et al. 1981; Szumilo et al. 1986). Purified α-glucosidase I from S. cerevisiae (Bause et al. 1986), plant (Zeng & Elbein 1998), and different mammalian tissues (Bause et al. 1986; Bause et al. 1989; Shailubhai et al. 1987; Zeng & Elbein 1998) exhibit comparable biochemical properties such as the same unique substrate specificity and similar sensitivity for inhibitors (Grinna & Robbins 1980; Saunier et al. 1982; Zeng & Elbein 1998). Protein sequences derived from the cDNA encoding yeast and human α-glucosidase I exhibited regions of significant amino acid identity (Romero et al. 1997). All together, these similarities suggest that α-glucosidase I has been conserved among eukaryotes.

So far several functions have been proposed for α-glucosidase I including a regulatory role during transition of oligosaccharides from their lipid carrier to the protein (Burda & Aebi
controlling the flux of free oligosaccharides (Durrant & Moore 2002), and most importantly, participating in the ER quality control (QC). Inhibition of α-glucosidase I interferes with further processing of oligosaccharides by α-glucosidase II which consequently can affect glycoprotein folding and QC. In higher eukaryotes impairing α-glucosidase I activity (in vivo) causes severe dysfunction and even death. Disruption of α-glucosidase I in Arabidopsis resulted in abnormal accumulation of storage proteins and seed development (Boisson et al. 2001). It is also reported that a defect in α-glucosidase I activity is fatal in humans (De Praeter et al. 2000). It has been observed that α-glucosidase I inhibitors possess potential therapeutic applications, particularly against the viruses in which N-linked glycoproteins are essential for the infectivity and life cycle (Lu et al. 1995; Mehta et al. 2002). However, despite the important roles of α-glucosidase I and the potential therapeutic applications of its inhibitors, it is one of the least characterized glycoside hydrolases among the ER-processing glycosidases. Therefore, this thesis was primarily focused on purification and characterization of α-glucosidase I from S. cerevisiae. In this regard a purification method for the soluble α-glucosidase I was established. A catalytically active fragment of enzyme was isolated, identified, and characterized. Expression of the catalytic region was investigated, and the effect of deletion of other regions (i.e. predicted N-terminal and transmembrane domain) of α-glucosidase I on functional expression and enzyme properties (e.g. specific activity and glycosylation) was evaluated. Important residues of the enzyme were identified (e.g. potential binding residues). Moreover, the importance of two highly conserved carboxylic acid residues residing in the catalytic region of the enzyme (E613 and D617) was examined.
1.2 ENDOMERIC RETICULUM AS THE CORE OF N-GLYCOSYLATION

In eukaryotic cells, a wide variety of proteins such as fractions of the plasma membrane and cell surface proteins, as well as secretory proteins, are translocated into the rough ER (Kostova & Wolf 2003). Translocation of proteins across the membrane into the ER takes place through a complex protein channel which is called the translocon (Johnson & van Waes 1999). The translocon directs polypeptide chains into the lumen or inserts them into the membrane of the ER (Rapoport et al. 1996). Translocated proteins are usually in an unfolded state. Therefore, the main function of the ER is to help proteins acquire their native conformation by employing different molecular mechanisms such as protein folding assisted by N-glycosylation (Kostova & Wolf 2003). In the ER, proteins are exposed to a complex environment containing enzymes and molecular chaperones involved in N-glycosylation, glycoprotein folding, and QC (Chevet et al. 2001). Concurrent with translocation of the growing polypeptide chain from the ribosome into the ER, oligosaccharyl transferase (OST) assembles the oligosaccharide on the protein (Johnson & van Waes 1999). Soon after, the newly assembled N-glycan undergoes stepwise processing with an array of glycosyl hydrolases (Herscovics 1999a). Processing of N-glycans in conjunction with chaperones and other ER enzymes enable N-glycoproteins to acquire their final native conformation (Helenius & Aebi 2004). The assembly and processing of oligosaccharides in the ER, as the early steps of N-glycosylation, are highly conserved events among eukaryotes (Robbins 1999). Figure 1.1 summarizes the early stages of N-glycosylation in the ER.
Figure 1.1. Schematic diagram of N-glycosylation events in a eukaryotic cell. Figure shows formation, assembly, and processing of the oligosaccharide in the rough ER; adapted from Helenius and Aebi (2002).
1.3 ASSEMBLY OF THE OLIGOSACCHARIDE

Assembly of the oligosaccharide on the protein occurs in the rough ER. Formation of an \(N\)-glycosidic bond requires an oligosaccharide linked to a lipid carrier as a donor, an Asn residue situated in an appropriate sequence of a polypeptide chain as an acceptor, and the OST which transfers the oligosaccharide onto the recognized nascent sequence of the polypeptide chain (Kornfeld & Kornfeld 1985).

1.3.1 Oligosaccharide precursor

In the early 1970’s, it was shown that liver microsomes from chick embryo were able to synthesize lipid-linked oligosaccharides containing glucose which were then transferred to a protein (Parodi et al. 1972). Thereafter, Kornfeld and co-workers (1978) proposed that the tri-antennary 14 sugar oligosaccharide, Glc\(_3\)Man\(_9\)GlcNAc\(_2\) (Fig 1.2), was the moiety that transfers \textit{en bloc} to the Asn residue of a polypeptide chain from its lipid-linked carrier, dolichol-pyrophosphate (Dol-P-P), in virally infected cells (Li et al. 1978). This finding was concurrent with the isolation of Glc\(_3\)Man\(_9\)GlcNAc\(_2\) from lower and higher eukaryotic cells (Hubbard & Robbins 1979; Parodi 1979), except in some protozoa species (Katyal et al. 1984; Parodi 1993), suggesting it is a well conserved structure in almost all eukaryotes. Furthermore, it was established that Glc\(_3\)Man\(_9\)GlcNAc\(_2\) is the common oligosaccharide which is predominantly transferred \textit{in vivo} to proteins from its lipid carrier (Kornfeld & Kornfeld 1985; Parodi & Leloir 1979; Tanner & Lehle 1987).

\textit{In vivo} biosynthesis of Glc\(_3\)Man\(_9\)GlcNAc\(_2\)-P-P-Dol begins on the cytoplasmic side of the ER membrane with addition of an \(N\)-acetylglucosamine (GlcNAc)-phosphate from its nucleotide sugar donor (UDP-GlcNAc) to dolichyl-phosphate (Dol-P). This reaction is followed by addition of a second GlcNAc from UDP-GlcNAc and then five mannose
molecules from GDP-mannose (GDP-Man), to form Man$_3$GlcNAc$_2$-P-P-Dol. Subsequently, Man$_3$GlcNAc$_2$-P-P-Dol as well as Dol-P- mannose (Dol-P-Man) and Dol-P-glucose (Dol-P-Glc) are flipped from the cytoplasmic side into the luminal side of the ER membrane (Burda & Aebi 1999; Helenius & Aebi 2002). The remaining four mannose and three glucose residues are added to Man$_3$GlcNAc$_2$-P-P-Dol from Dol-P-Man and Dol-P-Glc in a stepwise manner (Figure 1.1) (Burda et al. 1996). The final structure of the oligosaccharide linked to its lipid carrier before en bloc transferring to the selected Asn residue of a polypeptide is a tri-antennary tetradecasaccharide, Glc$_3$Man$_9$GlcNAc$_2$-P-P-Dol (Figure 1.2).

In vitro studies suggested that a lipid-linked oligosaccharide is transferred more rapidly to the protein when it carries glucose residues than an oligosaccharide intermediate devoid of glucose residues (Sharma et al. 1981; Spiro et al. 1979a; Turco et al. 1977). In contrast, removing mannose residues from the α-(1,6) arm of an oligosaccharide (Figure 1.2) using an α-mannosidase did not reduce the rate of transformation of truncated oligosaccharides to proteins (Spiro et al. 1979a; Staneloni et al. 1981).

Studies using genetic approaches provided more detailed information about the importance of glucose residues for efficient transformation of oligosaccharides to proteins. For instance, a S. cerevisiae mutant unable to synthesize Dol-P-Glc, showed decreased efficiency of glycosylation of proteins (Ballou et al. 1986). In addition, studies on asparagine-linked glycosylation defective mutants (alg) of S. cerevisiae revealed more detailed information about the role of glucose residues in the transferring of oligosaccharides to proteins. The alg6 and alg8 mutants which were unable to transfer the first and second α-(1,3) glucose residues to the oligosaccharide core accumulated Man$_9$GlcNAc$_2$-P-P-Dol, and Glc$_1$Man$_9$GlcNAc$_2$-P-P-Dol, respectively (Huffaker & Robbins 1983; Runge & Robbins 1986). Furthermore, it was indicated that the alg10 mutant accumulated Glc$_2$Man$_2$GlcNAc$_2$-
P-P-Dol demonstrating that the last glucose residue, α-(1,2) glucose, did not transfer to the oligosaccharide (Burda & Aebi 1998). Therefore it was established that glucose residues are essential for efficient \( N \)-glycosylation. In humans, deficiency in the transferring of glucose residues to the oligosaccharide core results in hypoglycosylation of glycoproteins and ultimately gives rise to multi-systemic disorders, which are generally recognized as congenital disorder of glycosylation (CDG) (Chantret et al. 2003; Imbach et al. 1999). It is reported that mutations in the human homologues of \( S. \) cerevisiae \( ALG6 \) and \( ALG8 \) genes caused a neurological disorder and a gastrointestinal problem categorized as CDG type-Ic and CDG type-Ih, respectively (Chantret et al. 2003; Damen et al. 2004; Imbach et al. 1999).

Based on \textit{in vitro} studies that show removing α-(1,2) glucose from Glc\(_3\)Man\(_9\)GlcNAc\(_2\) was concomitant with reduction in transfer efficiency of oligosaccharide to protein (Murphy & Spiro 1981) and \textit{in vivo} studies in which an \textit{alg10} mutant of \( S. \) cerevisiae accumulated lipid-linked Glc\(_2\)ManGlcNAc\(_2\) (Burda & Aebi 1998), it was suggested that the terminal α-(1,2) glucose of Glc\(_3\)Man\(_9\)GlcNAc\(_2\) likely plays a key role in substrate recognition by OST and may serve as a signal for OST to indicate that the structure of oligosaccharide precursor is completed (Burda & Aebi 1998). However, it should be noted that occurrence of glucose residues in oligosaccharides are not an absolute requirement for efficient \( N \)-glycosylation in all eukaryotes as some protozoa species normally transfer an oligosaccharide devoid of glucose to the protein (Parodi 1993).
Figure 1.2. Structure of the common 14-sugar oligosaccharide precursor. $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ has a tri-antennary structure which is transferred \textit{en bloc} to the protein from its lipid carrier (Dol-P-P) during the assembly stage; adapted from Trombetta (2003).
1.3.2 Asn-X-Thr/Ser essential sequence for N-glycosylation

After the oligosaccharide acquires its complete lipid-linked structure, Glc₃Man₉GlcNAc₂ is transferred en bloc to the protein (Figure 1.1). It has been established that the Asn-X-Thr/Ser sequence is essential for the polypeptide to be N-glycosylated (Bause 1979; Pless & Lennarz 1977). In that sequence, X can be any amino acid except proline (Ronin et al. 1978). Although Asn-X-Thr/Ser is a ubiquitous sequence in proteins, not all of the Asn residues in this sequence are glycosylated. It was reported that 64.9% of the proteins in the Swiss-Prot database contain Asn-X-Thr/Ser (Apweiler et al. 1999). However, it was noted that only 65% of the potential N-glycosylation sites are occupied (Apweiler et al. 1999). This indicates that although the Asn-X-Thr/Ser is an essential sequence it is not sufficient for N-glycosylation. Factors that control effective use of an N-glycosylation site are not well understood (Marchal et al. 2003). However, factors both at the cellular and molecular levels likely affect N-glycosylation. Protein translation rate, availability and structure of the lipid-linked oligosaccharide, level of OST, and the ability of OST to efficiently recognize N-glycosylation sites along the protein chain during translation are likely important (Helenius & Aebi 2004; Petrescu et al. 2004).

1.3.3 Oligosaccharyl transferase

OST is a heteromeric ER membrane-bound enzyme which has a high degree of structural conservation, and is found in lower and higher eukaryotes (Knauer & Lehle 1999; Silberstein & Gilmore 1996). The function of the OST, is transferring the oligosaccharide from its lipid-linked carrier to the Asn residue of the nascent sequence of polypeptide, is vital for the viability of eukaryotes (Silberstein & Gilmore 1996). Recent studies on S. cerevisiae OST demonstrated that the Stt3p subunit, which is highly conserved in all OST, contains the
active site, and is faced to the open front of the translocon pore (Kim et al. 2005; Yan et al. 2005). This structural configuration allows OST to assemble an oligosaccharide on the Asn residue as soon as the newly synthesized nascent sequence of polypeptide chain enters the ER. It seems that an oligosaccharide can be transferred to an Asn residue of Asn-X-Thr/Ser when it is 12-14 residues from the transmembrane segment of the protein (Nilsson & von Heijne 1993).

*In vitro* studies with two secretory glycoproteins, ovalbumin and RNase, showed that oligosaccharide can be transferred to Asn-X-Thr/Ser of the denatured form of these proteins and not the native form which suggests that this is a co-translational modification (Pless & Lennarz 1977). Other studies on the $N$-glycosylation of Bβ and γ chains of rat fibrinogen showed that glycosylation happens co-translationally for the γ chain and co-translationally or early post-translationally for the Bβ chain (Nickerson & Fuller 1981). Recently, it was reported that $N$-glycosylation of human coagulation factor VII takes place post-translationally (Bolt et al. 2005). In addition, $N$-linked glycans within 10 residues of the C-terminal of some proteins have been reported suggesting that in these proteins $N$-glycosylation likely takes place post-translationally (Ben Dor et al. 2004). Therefore although not conclusively established, it is speculated that $N$-glycosylation predominantly takes place co-translationally before a protein acquires its final conformation.

### 1.4 PROCESSING OF $N$-GLYCANS

Soon after the transfer of Glc$_3$Man$_9$GlcNAc$_2$ to the protein, all glucose residues and some mannose residue(s) are sequentially removed in the ER. This trimming process is administrated by α-glucosidases (I and II) and ER mannosidase(s). The processing of $N$-
glycans takes place in a highly conserved manner in lower and higher eukaryotes (Herscovics 1999a; Herscovics 1999b).

### 1.4.1 Processing α-glucosidases (I and II)

Early studies showed there was a glucosidase activity associated with hen oviduct cell membrane, which hydrolyzed glucose residues from the newly transferred glucose-containing oligosaccharide that was bound to S-carboxymethylated alpha-lactalbumin (Chen & Lennarz 1978). Kornfeld and co-workers (1978) similarly demonstrated that the newly assembled oligosaccharide of vesicular stomatitis virus G glycoprotein in virally infected Chinese hamster ovary (CHO) cells is “processed” by removing the glucose residues with subsequent removal of mannose residues from outer branches (Kornfeld et al. 1978; Li et al. 1978). Spiro et al. (1979b) described a microsomal glucosidase activity isolated from calf thyroid which could selectively remove glucose residues from oligosaccharides containing glucose. Based on the unique biochemical properties of this enzyme it was suggested that it was a distinct activity from other glucosidase previously isolated from thyroid (Spiro et al. 1979b).

Hubbard and Robbins (1979) showed that glucose residues are removed from Glc₃Man₉GlcNAc₂ linked to protein in a stepwise manner. Indeed, it was established that the outermost glucose is removed rapidly, the second glucose residue more slowly, and the innermost glucose residue, most slowly (Hubbard & Robbins 1979; Jakob et al. 1998a). Moreover, it was demonstrated that two distinct glucosidase activities participated in the early steps of processing. They were identified as α-(1,2)-glucosidase or α-glucosidase I, which removes the distal glucose residue from the oligosaccharide, and α-(1,3)-glucosidase or α-glucosidase II, which cleaves the two α-(1,3) glucose from the Glc₂Man₉GlcNAc₂
(Grinna & Robbins 1979; Grinna & Robbins 1980). Subsequently, processing α-glucosidase I and II were isolated from lower and higher eukaryotes, and orthologs showed comparable biochemical properties (Bause et al. 1989; Burns & Touster 1982; Kilker, Jr. et al. 1981). The respective genes were identified and cloned, and orthologs showed a high degree of sequence homology (Kalz-Fuller et al. 1995; Romero et al. 1997; Trombetta et al. 1996). The only eukaryotes that do not have α-glucosidase I activity are some protozoan species, i.e. trypanosoma (Bosch et al. 1988). This indicates that removing glucose residues at the beginning of trimming process is a highly conserved incident among most eukaryotes.

1.4.2 Processing α-mannosidase(s)

Removing all glucose residues from the α-(1,3) arm of Glc₃Man₉GlcNAc₂ linked to the protein is followed by cleavage of at least one distal α-(1,2) mannose residue(s) from the α-(1,6) arm of Man₉GlcNAc₂ (Herscovics 1999a; Jelinek-Kelly & Herscovics 1988; Kornfeld & Kornfeld 1985). The ER α-(1,2)-mannosidase is the last trimming glycosyl hydrolase that normally processes the N-glycans devoid of glucose residues before the N-linked glycoproteins are transferred to the Golgi. Here the truncated N-glycans undergo further modification by the Golgi-mannosidase and other glycosyl transferases to form mature structures (Herscovics 1999a; Kornfeld et al. 1978). The processing of the N-glycan by α-glucosidases (I and II) and an α-mannosidase in the lumen of the ER is summarized in Figure 1.1. Although cleavage of α-(1,2) mannose normally occurs after removing glucose residues, trimming of glucose is not a prerequisite for the ER α-(1,2)-mannosidase activity (Jakob et al. 1998b; Lodish & Kong 1984). Indeed, in mutated plant and mammalian cells lacking α-glucosidase I activity (i.e. Lec23 Chinese hamster ovary cells) it was shown that mannose residues from triglucosylated oligosaccharide were removed and generated Glc₃Man₇.
$\text{GlcNAc}_2$ demonstrating glucose trimming is not essential for the ER $\alpha$-mannosidase activity (Boisson et al. 2001; Hong et al. 2004).

It should be noted that the function of processing glycosidases in the ER is not simply to provide trimmed $N$-glycans for subsequent modifications in the Golgi (Trombetta 2003). In mutated $S. \text{cerevisiae}$ for instance, inability to remove glucose residues did not interfere with further modification of $N$-glycans in the Golgi (Esmon et al. 1984). Providing specific signal epitopes which can be selectively recognized by other molecular mechanisms involved in folding and degradation of proteins in the ER is suggested as the primary function of processing glycosidases (Helenius & Aebi 2004).

1.4.3 Role of glucose residues of $N$-glycans in quality control

The protein QC in the ER is a multifaceted process which provides stringent conditions to ensure only properly folded proteins can leave the ER (Ellgaard et al. 1999). Proteins that can not acquire their active conformation are retained in the cells and degraded by the ER associated degradation system (ERAD), which involves retrograde transfer of proteins from the ER to the cytosol followed by ubiquitin-mediated degradation by the proteasome (Yoshida 2003). Glucose residues of $N$-linked glycoproteins play a crucial role for $N$-glycoproteins QC in the ER and serve as a bio-recognition signal showing the folding status of $N$-glycoproteins (Helenius & Aebi 2004).

After formation of $\alpha$-$\text{Glc}(1,3)\text{Man}_9\text{GlcNAc}_2$ linked-protein by sequential trimming with $\alpha$-glucosidase I and II, truncated monoglycosylated oligosaccharide linked-proteins interact with the ER residence lectin chaperones, calnexin (a type I transmembrane protein) and/or calreticulin (a soluble luminal ER protein) in higher eukaryotes, and Cne1p (a calnexin homologue) in $S. \text{cerevisiae}$ (Xu et al. 2004), to facilitate folding of proteins.
Di- and triglucosylated glycoproteins do not bind to calnexin and calreticulin (Spiro et al. 1996; Ware et al. 1995). It was also reported that monoglucosylated oligosaccharides can serve as a signal for protein folding in *S. cerevisiae* (Jakob et al. 1998b). These results together, suggest an integral function for α-glucosidase I and II in mediating interaction of the newly assembled *N*-glycans with lectin chaperones.

After binding Glc₃Man₉GlcNAc₂ linked-protein to the lectin chaperone, a series of molecular mechanisms are involved to assist proteins to acquire their final conformation. For instance, ERp57 a thiol-dependent reductase in conjunction with the molecular chaperone complex, assists protein folding by facilitating the formation of disulfide bonds (Elliott et al. 1997; Van der Wal et al. 1998). It was shown that binding ERp57 to a glycoprotein was also dependent on glucose trimming, as mutant cells deficient in α-glucosidase I activity did not interact with ERp57 (Van der Wal et al. 1998).

Subsequently, α-glucosidase II removes the last glucose of monoglucosylated *N*-glycans which results in the release of protein from the chaperones. At this stage UDP-Glc: glycoprotein glucosyltransferase (GT) may selectively re-glucosylate misfolded proteins which helps the protein re-bind to the lectin chaperones (Helenius & Aebi 2004; Parodi 2000b). GT activity has been reported from different eukaryotes (Parker et al. 1995; Sousa et al. 1992; Trombetta et al. 1989) except *S. cerevisiae* (Jakob et al. 1998b). The de- and re-glucosylation cycle continues until proteins are either properly folded or degraded by the ERAD.
1.5 OTHER PROPOSED FUNCTIONS FOR α-GLUCOSIDASE I

In addition to the role of processing α-glucosidase I in protein folding and QC as described earlier, the following functions are suggested to occur during the N-glycosylation process.

OST possesses a hydrolytic activity that cleaves Glc₃Man₉GlcNAc₂ from its dolichol pyrophosphate carrier when the accessibility to nascent peptides is limited (Anumula & Spiro 1983; Spiro & Spiro 1991). It has also been shown that the ER α-glucosidase I can hydrolyze the terminal α-(1,2) glucose from Glc₃Man₉GlcNAc₂-P-P-Dol. In vitro studies suggest that Glc₂Man₉GlcNAc₂ can not be hydrolyzed from its lipid-linked carrier by OST. In addition, it is shown that a glucosyl-transferase is able to re-glucosylate Glc₂Man₉GlcNAc₂-P-P-Dol. Therefore, it is suggested that ER processing α-glucosidase I may have a regulatory role for adjusting the level of the oligosaccharide precursor through a glucosyl-transferase:glucosidase I cycle (Spiro & Spiro 1991).

The terminal α-(1,2) glucose residue plays a key role for oligosaccharide recognition by OST as mentioned in section 1.3.1 (Burda & Aebi 1998). It has also been established that the terminal α-(1,2) residue is hydrolyzed rapidly from the protein-linked oligosaccharide by α-glucosidase I immediately after transferring to a nascent polypeptide (Hubbard & Robbins 1979; Jakob et al. 1998a). Thus, the key glucose for substrate recognition by the OST is removed very quickly from the N-linked oligosaccharide to prevent the interaction of the OST complex with its product. This hinders any possible further reverse hydrolytic reaction by OST. Therefore, it has been suggested α-glucosidase I may protect the newly assembled N-glycans from being hydrolyzed by OST (Burda & Aebi, 1998).

In cells with inhibited α-glucosidase I activity, the level of free Glc₃Man₉GlcNAc₂ and Glc₃Man₉GlcNAc increased (Durrant & Moore 2002). This increase in free
oligosaccharide is likely related to the hydrolytic activity of OST, which removes the Glc₃Man₉GlcNAc₂ from its lipid carrier (Spiro & Spiro 1991) and/or the ER luminal N-glycanase, which cleaves the Glc₃Man₉GlcNAc from misfolded glycoproteins (Spiro & Spiro 2001). This indicates that α-glucosidase I activity is likely required for controlling the flux, and disposing of, the free glucosylated-oligosaccharide produced during N-glycan biosynthesis (Durrant & Moore 2002). Indeed, this function seems crucial in vivo at least for humans. In a patient deficient in α-glucosidase I activity, the free tetrasaccharide Glc₃Man was accumulated in the urine (De Praeter et al. 2000), suggesting the importance of α-glucosidase I in disposing of free oligosaccharide containing α-(1,2)-linked glucose in vivo (Durrant & Moore 2002).

1.6 INHIBITION OF PROCESSING α-GLUCOSIDASES

1.6.1 In vitro studies

Several studies have shown that inhibition of N-glycan processing glucosidases affects secretion and maturation of a large number of glycoproteins. In this regard, it was reported that rat intestinal epithelial cells (IEC-6) treated with 1-deoxynojirimycin (DNM), a glucose analog which acts as a common inhibitor for processing α-glucosidases, reduced cell surface glycopeptides by 60% (Saunier et al. 1982). Further studies with human hepatoma HepG2 cells treated with DNM indicated that α1-antitrypsin and α1-antichymotrypsin accumulated in the ER, which suggests that processing α-glucosidases are required for the effective transportation of these secretory glycoproteins from the ER to other destinations, i.e. the Golgi (Lodish & Kong 1984). Similar results were also observed from rat hepatocytes treated with DNM (Gross et al. 1983). Subsequently, it was shown that DNM and alkylated DNM derivatives, which have more inhibitory potency toward processing α-
glucosidase I, increased the intracellular accumulation of α1-antitrypsin with glucosylated-
oligosaccharide in treated HepG2 cells (Tan et al. 1991). Interestingly, in studies with α1-
antitrypsin, it was found that inhibition of processing α-glucosidases did not completely
block maturation of oligosaccharides. It was also shown that HepG2 cells treated with
castanospermine, an alkaloid inhibitor which is somewhat effective for processing α-
glucosidase I, impeded the secretion rate of glycoproteins α1-antitrypsin and caeruloplasmin
(Sasak et al. 1985). Furthermore, other studies indicated that castanospermine-treated cells
reduced interaction of glycoproteins with lectin chaperones (i.e. calnexin) which promoted
degradation of some glycoproteins receptors such as the α β T-cell antigen, nicotinic
acetylcholine, and thyrotropin (Kearse et al. 1994; Keller et al. 1998; Siffroi-Fernandez et al.
2002). Also, it was suggested that castanospermine likely affects cell-cell interaction by
reducing the expression of certain membrane glycoproteins which are required for cell
interactions (Grochowicz et al. 1996). Further in vitro studies indicated that impairing
processing α-glucosidase I and II activities using genetic approaches did not compromise cell
viability in lower eukaryotes (i.e. S. cerevisiae) and mammalian cultured cells. In these
studies, cells with compromised α-glucosidase I activity accumulated triglucosylated N-
glycans while cells lacking α-glucosidase II activity accumulated diglucosylated N-
oligosaccharide (D'Alessio et al. 1999; Esmon et al. 1984; Jakob et al. 1998b; Ray et al.
1991; Reitman et al. 1982).

1.6.2 Golgi endomannosidase an alternative route for processing of N-glycans

In vitro studies show that abolishing ER processing α-glucosidase I and II activities
affects overall glycoprotein synthesis but does not have a major impact on the cell viability,
or maturation and secretion of certain glycoproteins. For instance, α1-antitrypsin can be
partially secreted and gain its mature oligosaccharide without processing α-glucosidases suggesting that there is an alternative route that can process oligosaccharide (Moore & Spiro 1990; Rabouille & Spiro 1992). This expands the QC of N-glycosylation to the Golgi (Zuber et al. 2000).

Lubas and Spiro reported an alternative glucosidase-independent processing route for selective hydrolysis of oligosaccharide containing glucose carried out by Golgi endomannosidase (Lubas & Spiro 1987; Lubas & Spiro 1988). Endomannosidase is able to hydrolyze the \( \text{Glc}(3-1)\text{Man}_9\text{GlcNAc}_2 \) attached to the polypeptide or the lipid carrier by removing \( \text{Glc}(3-1)\text{Man} \) which yields \( \text{Man}_8\text{GlcNAc}_2 \) (Figure 1.3). The affinity of endomannosidase for processing of glucosylated oligosaccharides is affected by the number of glucose residues. \( \text{GlcMan}_9\text{GlcNAc}_2 \) is a preferred substrate for endomannosidase whereas \( \text{Glc}_2\text{Man}_9\text{GlcNAc}_2 \) or \( \text{Glc}_3\text{Man}_9\text{GlcNAc}_2 \) are processed at a slower rate (Lubas & Spiro 1988; Tulsiani et al. 1990). Moreover, processing α-glucosidase I and II inhibitors, DNM and castanospermine, did not inhibit the Golgi endomannosidase activity (Bause & Burbach 1996; Lubas & Spiro 1988). However, the \( \text{Man}_8\text{GlcNAc}_2 \) isomer produced by the ER α-mannosidase in which the outermost mannose from the middle branch (α-(1,3) branch) of the α-(1,6) arm is removed, differs from the \( \text{Man}_8\text{GlcNAc}_2 \) isomer formed with Golgi-endomannosidase in which the middle mannose of α-(1,3) arm, is cleaved (Figure 1.3). The \( \text{Man}_8\text{GlcNAc}_2 \) isomer from ER-processing glycosidases can be converted to \( \text{Man}_5\text{GlcNAc}_2 \) by Golgi α-mannosidase whereas the \( \text{Man}_8\text{GlcNAc}_2 \) from endomannosidase only can convert to \( \text{Man}_6\text{GlcNAc}_2 \) due to the inability of Golgi α-mannosidase to remove the outermost α-(1,2)-linked mannose from the middle branch of the α-(1,6) arm of \( \text{Man}_9\text{GlcNAc}_2 \) (Figure 1.3) (Lubas & Spiro 1988). Although the Golgi endomannosidase can process newly assembled \( \text{Glc}_3\text{Man}_9\text{GlcNAc}_2 \), its affinity toward \( \text{GlcMan}_9\text{GlcNAc}_2 \) makes it less effective as
an alternative processing route when ER processing α-glucosidase I is blocked. As well, formation of a specific Man₈GlcNAc₂, isomer which differs from the polymannose oligosaccharide product of ER α-mannosidase, may affect further essential modification of some oligosaccharides in the Golgi. Unlike processing α-glucosidase I and II which are widely found among eukaryotes, Golgi endomannosidase is limited to some higher eukaryotes and this enzyme is absent in invertebrates and yeast (Dairaku & Spiro 1997).
Figure 1.3. Schematic diagram of the Golgi endomannosidase pathway.
1.6.3 In vivo studies

In contrast to in vitro studies that indicate cells with compromised processing α-glucosidases activities are viable and do not show any discernable phenotype, an in vivo deficiency of the processing α-glucosidase I and II in multicellular eukaryotes causes severe physiological and growth disorders. For example, Arabidopsis mutants deficient in α-glucosidase I activity were found to produce abnormal shrunken seeds. Mutant seeds accumulated a low content of storage proteins, had abnormal cell enlargement, and difficulties in cellulose biosynthesis and embryo development (Boisson et al. 2001; Gillmor et al. 2002). However, the role of α-glucosidase I in cellulose biosynthesis is indirect since the catalytic subunits of cellulose synthase are not N-glycosylated (Gillmor et al. 2002). In humans, mutations in processing α-glucosidase I (classified as CDG type-IIb) led to inactivation of the enzyme and were fatal for a neonate (De Praeter et al. 2000; Volker et al. 2002).

Moreover, in vivo studies have indicated that growth abnormality and physiological defects occur in higher eukaryotes when α-glucosidase II activity is abolished. For instance, disruption of the gene that encodes processing α-glucosidase II in Dictyostelium discoideum, a soil-living amoeba, caused abnormal growth (Freeze et al. 1997). In Arabidopsis mutants deficient in α-glucosidase II activity, a reduced rate of cell division and abnormal root development were observed (Burn et al. 2002). Finally, in humans, it was reported that mutation in processing α-glucosidase II is associated with autosomal dominant polycystic liver disease (Drenth et al. 2003; Li et al. 2003).
1.7 CONTROLING VIRAL INFECTIVITY BY INHIBITION OF α-GLUCOSIDASES

Enveloped viruses are covered by a membrane that contains proteins from the host-cell called “envelope proteins”, which are essential for virus assembly and entry. The major constituent of envelope proteins are N-linked glycoproteins that play an important function in the stability, antigenicity, and biological function of the virus (Goffard & Dubuisson 2003); therefore, inhibition of N-glycosylation can likely affect the life cycle. Hence, inhibitors of ER processing α-glucosidases can be employed as potential broad range anti-viral agents (Mehta et al. 1998). Indeed, several studies have shown that hindering the activity of ER processing α-glucosidases reduces viral infectivity. Studies on lymphocytes infected with human immunodeficiency virus (HIV), an envelope virus, revealed that the functional properties of the membrane-associated envelope glycoproteins were modified when infected cells were treated with DNM. DNM inhibited the envelope glycoproteins-mediated membrane fusion process, which resulted in reduced infectivity (Papandreou et al. 2002). In vitro studies have also shown that N-butyl-DNM (NB-DNM), a potent processing α-glucosidase I inhibitor interfered with the synthesis of gp120 (Karlsson et al. 1993), a heavily N-glycosylated envelope glycoprotein which is essential for entry and infectivity of HIV (Greenberg et al. 2004). HIV infectivity was likewise reduced in cells treated with a 6-butanoyl derivative of castanospermine, a processing α-glucosidase I inhibitor, due to conformational changes of the expressed mature envelope glycoproteins (e.g. gp120) (Taylor et al. 1994). In most of these studies, the triglucosylated oligosaccharide was the predominant form of the N-linked oligosaccharide, and castanospermine was more effective than DNM against HIV. Therefore, it was speculated that blocking α-glucosidase I activity can efficiently impede viral infectivity (Asano et al. 1995). Successful reduction of
infectivity by the inhibition of ER processing α-glucosidases was also reported for other viruses such as bovine viral diarrhea (Jordan et al. 2002), flavivirus (Wu et al. 2002), and pig endogenous retrovirus (Hazama et al. 2003).

Although inhibition of processing α-glucosidases, especially α-glucosidase I, attenuated the infectivity of enveloped viruses, there are several drawbacks associated with their practical application as a drug. These include the non-selective nature of inhibitors of ER processing α-glucosidases (i.e. DNM) (Papandreou et al. 2002), difficulties in achieving the therapeutic serum concentration required for inducing the inhibitory effect (Mehta et al. 1998), and adverse physiological effects of α-glucosidase inhibitors on N-glycosylation and other glycosidases of the normal cell (Asano 2003). Such problems have limited the use of currently available inhibitors as an effective drug. Therefore, there is a need for inhibitors with more specificity and potency which can specifically block only ER processing α-glucosidases. A drug specific for α-glucosidase I would likely have more profound effects on controlling viral infectivity (Butters et al. 2000). However, since the mechanism and characteristics of processing α-glucosidase I are not very well understood, such specific inhibitors have not yet been designed.

1.8 **GENERAL CHARACTERISTICS OF α-GLUCOSIDASE I**

1.8.1 **Classification and nomenclature**

Glycosyl hydrolases have been classified into 100 families at the current time based on their amino acid sequence similarities. Information about these families is available at the Carbohydrate-Active-enZYme server (CAZy), URL: http://afmb.cnrs-mrs.fr/CAZY/ (Coutinho & Henrissat 1999). Based on this classification, processing α-glucosidase I orthologs are placed in the unique family 63 of glycosyl hydrolases. Also, according to the
ENZYME database (URL: http://www.expasy.org/enzyme/), which assigns the name of enzymes based on the recommendations of the nomenclature committee of the International Union of Biochemistry and Molecular Biology (IUBMB) (Bairoch 2000), the official name of α-glucosidase I is “mannosyl-oligosaccharide glucosidase” with the enzyme commission (EC) number 3.2.1.106.

1.8.2 Biochemical properties

α-Glucosidase I, isolated from different eukaryotes e.g. yeast, plant and mammalian cells, possesses comparable biochemical properties (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). α-Glucosidase I in all eukaryotes excises specifically the distal α-(1,2)-linked glucose residue from the α-(1,3) arm of the natural substrate Glc3Man9GlcNAc2, and does not show any activity toward the α-(1,3)-linked glucose from Glc(2,1)Man9GlcNAc2 (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). Moreover, α-glucosidase I does not exhibit any aryl α- and β-glucosidase activities. It does not require any divalent metal ions for activity since the activity is not inhibited by metal chelating agents such as EDTA and O-phenanthroline (Bause et al. 1986; Bause et al. 1989; Shailubhai et al. 1987). It was reported that heavy metal ions such as Co^{2+}, Zn^{2+}, Ag^{+} and Hg^{2+} have an inhibitory effect on α-glucosidase I isolated from bovine mammary glands (Shailubhai et al. 1987). The inhibitory effect of heavy metal ions on mammalian α-glucosidase I was shown to be prevented using an excess amount of dithiothreitol (Shailubhai et al. 1987), suggesting involvement of a sulfhydryl group in the mammalian enzyme catalytic activity (Pukazhenthi et al. 1993a). It was also reported that Tris at 50 mM inhibited yeast α-glucosidase I (Saunier et al. 1982). The optimum pH for α-glucosidase I activity for all orthologs was determined to
be near neutral pH, suggesting an non-lysosomal origin for this enzyme (Bause et al. 1986; Bause et al. 1989; Shailubhai et al. 1987; Zeng & Elbein 1998).

1.8.3 Substrate specificity of α-glucosidase I

Besides the specificity of α-glucosidase I orthologs towards the α-(1,2)-linked glucose, other structural features of the oligosaccharide likely have an effect on the catalytic activity. For example, α-glucosidase I from rat liver has shown the highest activity towards free oligosaccharides, lower for lipid-linked and the lowest for peptide-linked oligosaccharides (Grinna & Robbins 1980). Similarly α-glucosidase I isolated from mung beans showed the highest activity towards free 14 sugar oligosaccharide, lower for the peptide-linked, and the lowest for protein-linked oligosaccharide (Zeng & Elbein 1998). Furthermore, it was shown that removing mannose residue(s) from the α-(1,6) arm of poly-mannose 14 sugar oligosaccharide precursor had different effects on α-glucosidase I activity from yeast, plant and mammalian sources. It was reported that α-glucosidase I activity from rat liver dropped 90% when Glc₃Man₅GlcNAc was used instead of Glc₃Man₉GlcNAc (Grinna & Robbins 1980). In contrast to mammalian α-glucosidase I activity, that from S. cerevisiae was slightly stimulated by removing mannose residues from the α-(1,6) arm of poly-mannose (Saunier et al. 1982). This stimulatory effect was also reported for the plant α-glucosidase I activity when truncated oligosaccharides, Glc₃Man₁₅(6,7)GlcNAc, were used as the substrate (Zeng & Elbein 1998). Interestingly, the decrease and/or increase in α-glucosidase I activity was proportional to the number of mannose residues of the oligosaccharide substrate.

Although the α-(1,2) glycosidic bond between the two glucose units of the oligosaccharide is the cleavage site of α-glucosidase I, this enzyme is not able to catalyze the
disaccharide kojibiose (α-D-Glc-(1,2)-α-D-Glc). Interestingly, it was reported kojibiose had an inhibitory effect on α-glucosidase I isolated from yeast, plant, and mammalian sources when Glc$_3$Man$_9$GlcNAc was used as the substrate (Bause et al. 1986; Shailubhai et al. 1987; Zeng & Elbein 1998).

1.8.4 Domain orientation

Hydrophobicity analysis of the amino acid sequences of selected α-glucosidase I orthologs e.g. *S. cerevisiae*, *Arabidopsis thaliana* (*A. thaliana*), *Caenorhabditis elegans* (*C. elegans*), and *Homo sapiens* (*H. sapiens*) indicates that these orthologs have the highest hydrophobicity at the region close to the N-terminal end of the protein. This suggests that this short hydrophobic region likely serves as the transmembrane domain, linking a short N-terminal domain to a large C-terminal domain (Figure 1.4). Indeed, this structural prediction is in agreement with the experimental findings that showed α-glucosidase I orthologs have the same domain orientations. From the early studies it became evident that α-glucosidase I is membrane-associated and isolated from the microsomes. Shailubhai et al. (1991) examined the trypsin accessibility of α-glucosidase I from rat mammary cells in undisrupted microsomes. They observed that hydrolysis of the sealed membrane by trypsin resulted in a shift in the subunit molecular mass from 85 kDa to 82 kDa without releasing activity from the membrane suggesting rat mammary α-glucosidase I has a short cytoplasmic domain (Shailubhai et al. 1991). The same observation was also reported for the human α-glucosidase I. It was determined that hydrolysis of the intact microsomes with trypsin was concomitant with a reduction of 4 kDa mass from the mass of the 95 kDa subunit of the recombinant human α-glucosidase I, thus indicating a short cytoplasmic oriented domain (Kalz-Fuller et al. 1995). Kalz-Fuller et al. (1995) using an immunolocalization technique,
showed that human α-glucosidase I is associated with the nuclear envelope and the ER of transfected mammalian cells. Furthermore, by employing a similar technique, it was noted that the Cwh41p (yeast α-glucosidase I) tagged by a hemagglutinin epitope is localized predominantly in the perinuclear-rim of transformed S. cerevisiae expressing Cwh41p, demonstrating it is an ER resident (Jiang et al. 1996). Therefore, it has been explicitly established that α-glucosidase I orthologs reside in the ER and have a short cytosolic N-terminal domain, a transmembrane anchoring region, and a large C-terminal domain, containing the catalytic domain, which is oriented towards the lumen of the ER (Jiang et al. 1996; Kalz-Fuller et al. 1995; Romero et al. 1997; Shailubhai et al. 1991). This pattern of domain orientation is the typical topological characteristic of the type II membrane protein residing in the ER.
Figure 1.4. The hydrophobicity profile of selected α-glucosidase I orthologs. The hydrophobicity score (Y axis) of amino acids was calculated over a window of 7-amino acid residues and plotted as a function of amino acid position (X axis) using Kyte and Doolittle method, available online at URL: http://ca.expasy.org/tools/protscale.html, for *S. cerevisiae* (A), *A. thaliana* (B), *C. elegans* (C) and *H. sapiens* (D). Arrows show the highest hydrophobicity region (Kyte & Doolittle 1982).
1.8.5 Orthologs are \(N\)-glycosylated

It has been reported that \(\alpha\)-glucosidase I from yeast, plant, and mammalian sources could bind to concanavalin A (ConA) (Bause et al. 1986; Bause et al. 1989; Zeng & Elbein 1998), a lectin protein with high binding affinity towards the trimannoside core of \(N\)-linked oligosaccharides (Chervenak & Toone 1995; Dam et al. 1998). Furthermore, treatment of \(\alpha\)-glucosidase I from plant and different mammalian sources with endo-\(\beta\)-N-acetylglucosaminidase H, which cleaves between the two GlcNAc residues of the \(N\)-glycans (Tarentino et al. 1978), was concomitant with decreasing the subunit molecular mass by approximately 2 kDa (Pukazhenthi et al. 1993b; Zeng & Elbein 1998). This clearly indicates that \(\alpha\)-glucosidase I orthologs are \(N\)-glycosylated and possibly carry a high mannose type oligosaccharide (Man\(_9\)GlcNAc\(_2\)) which has the mass of 1884 Da (Imperiali & O'Connor 1999). Prediction of \(N\)-glycosylation sites for the selected orthologs of \(\alpha\)-glucosidase I by NetGlyc server (URL: http://www.cbs.dtu.dk/services/NetGlyc/) revealed that all orthologs carry at least one potential \(N\)-glycosylation site (Figure 1.5). Interestingly, distribution, number, and probability of the \(N\)-glycosylation sites vary between eukaryotes (Figure 1.5). \(S.\) \textit{cerevisiae} \(\alpha\)-glucosidase I for instance, has 5 potential \(N\)-glycosylation sites with those located close to the transmembrane having a higher probability for being glycosylated than those near the C-terminus (Figure 1.5 A). In contrast to yeast, the human enzyme only has one predicted glycosylation site close to the C-terminus of the protein (Figure 1.5 D). Indeed, deglycosylation of recombinant human \(\alpha\)-glucosidase I resulted in a 2 kDa decrease in subunit molecular mass thus demonstrating the existence of only one \(N\)-glycosylation site (Kalz-Fuller et al. 1995).
Figure 1.5. Prediction of N-glycosylation site(s) of selected α-glucosidase I orthologs. The probability score of the N-glycosylation (Y axis) plotted versus the position of Asn position (at Asn-X-Thr/Ser) in the protein sequence (X axis) using NetNGlyc 1.0 server (URL: http://www.cbs.dtu.dk/services/NetGlyc/). (A) S. cerevisiae, (B) A. thaliana, (C) C. elegans and (D) H. sapiens.
1.9 INHIBITORS OF \( \alpha \)-GLUCOSIDASE I

As discussed in section 1.6, the inhibitors of ER processing \( \alpha \)-glucosidase I and II have been used to elucidate detailed information regarding the functions of the ER-trimming reactions and biological roles of specific \( N \)-glycan structures. Also, as described in section 1.7, \( \alpha \)-glucosidase I inhibitors may have potential therapeutic applications. One of the frequently used processing \( \alpha \)-glucosidases inhibitors is DNM, a glucose analog containing an endocyclic nitrogen instead of oxygen (Figure 1.6). DNM, 1,5-dideoxy-1,5-imino-D-glucitol, is an imino sugar that can be chemically synthesized (Comins & Fulp 1999) or isolated from natural sources such as mulberry leaves (Kim et al. 2003) and microorganisms such as the \( \textit{Bacillus} \) species (Stein et al. 1984). Saunier et al. (1982) were the first to demonstrate that DNM is a potent inhibitor for both \( \textit{S. cerevisiae} \) and mammalian processing \( \alpha \)-glucosidases (I and II). Further studies reported that 50% of \( \alpha \)-glucosidase I and \( \alpha \)-glucosidase II activities from calf liver were inhibited by 3 \( \mu \)M and 20 \( \mu \)M of DNM, respectively (Hettkamp et al. 1982). This clearly showed that DNM is not a specific inhibitor for only \( \alpha \)-glucosidase I. The Ki value of DNM was determined to be 16, 1 and 2.1 \( \mu \)M for \( \alpha \)-glucosidase I isolated from yeast, calf, and pig liver, respectively (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984). It was demonstrated that alkylated derivatives of DNM have a higher potency for inhibiting \( \alpha \)-glucosidase I than DNM. Alkylated DNM derivatives preferentially inhibit \( \alpha \)-glucosidase I. In this regard, the Ki value of \( N \)-methyl-DNM was reported as 0.3 \( \mu \)M and 0.07 \( \mu \)M for \( \alpha \)-glucosidase I from yeast and calf liver, respectively (Bause et al. 1986; Hettkamp et al. 1984). The Ki value of \( N \)-5'-carboxypentyl-DNM (CP-DNM) was determined to be 3 \( \mu \)M for yeast and 0.5 \( \mu \)M for calf and pig liver \( \alpha \)-glucosidase I, respectively (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984). Due to the significantly higher inhibitory effect of alkylated DNM in comparison to DNM, it was
suggested that the active site of α-glucosidase I may be located in a hydrophobic pocket (Bause et al. 1986). These results also clearly indicate that the yeast enzyme has a lower affinity toward DNM and alkylated derivatives than its mammalian counterpart.

Another potent inhibitor of α-glucosidase I is castanospermine (Figure 1.6). Castanospermine is an alkaloid (1,6,7,8-tetrahydroxyoctahydroindolizine) which is isolated from the seeds of the Australian chestnut tree, Castanosperum australe (Elbein 1991). It was reported that castanospermine, at approximately 1 μM could, inhibit 50% of the activity of α-glucosidase I from mung bean (Zeng & Elbein 1998).

Furthermore, some oligosaccharides also exhibit an α-glucosidase I inhibitory effect. For instance, it was reported the disaccharide kojibiose, α-D-Glc-(1,2)-α-D-Glc, can act as an inhibitor for processing α-glucosidases (Saunier et al. 1982). Kojibiose, at 1 mM, inhibited 76% of α-glucosidase I isolated from bovine mammary glands (Shailubhai et al. 1987). The Ki value for kojibiose was reported to be 95 μM and 55 μM for α-glucosidase I from pig liver and yeast, respectively (Bause et al. 1986; Bause et al. 1989). Nigerose, α-D-Glc-(1,3)-α-D-Glc, has a low inhibitory effect as at 1 mM only 20% of α-glucosidase I activity was inhibited (Shailubhai et al. 1987). Acarbose, a tetrasaccharide with a potent inhibitory effect for intestinal α-glucosidase and amylase, was ineffective at inhibiting processing α-glucosidase I isolated from calf liver (Hettkamp et al. 1984).
Figure 1.6. Structure of 1-deoxynojirimycin and castanospermine.
1.10 α-GLUCOSIDASE I ASSAY

One of the major constraints that hampers studies on α-glucosidase I is related to its unique specificity and the lack of any commercially available substrate for measuring α-glucosidase I activity. However, two methods have been developed to determine α-glucosidase I activity. The most frequently used method is based on a radiolabeled natural substrate and the other method is a spectrophotometric approach using a synthetic glucotriose. The principle of both methods is based on quantifying the amount of glucose released from the α-(1,2) linkage.

1.10.1 Radiolabeled assay

\[^{14}\text{C}]\) or \[^{3}\text{H}]\)glucose-labeled oligosaccharide substrate, obtained from microsomes or cell culture, has been frequently used for measuring α-glucosidase I activity. The radiolabeled substrate is generally prepared by metabolically labeling the oligosaccharide, is followed by isolation and purification of the oligosaccharide from the protein-linked or lipid-linked precursor (Grinna & Robbins 1979; Kilker, Jr. et al. 1981; Taylor et al. 1994). α-Glucosidase I activity is measured based on the quantity of released labeled glucose in the reaction. The released labeled glucose should be isolated from the remaining labeled mixture by different chromatography methods such as thin layer, gel-filtration, and ion exchange prior to determining the enzyme activity (Grinna & Robbins 1979; Kilker, Jr. et al. 1981; Taylor et al. 1994). Although this approach is very sensitive and can measure very low quantities of activity, it is labor intensive and time-consuming to obtain the purified labeled oligosaccharide substrate as well as isolate the labeled glucose product. However, the major drawback is the possibility of random labeling of glucose residues during biosynthesis of the oligosaccharide (Grinna & Robbins 1979). This could give rise to inequity and inconsistency.
in measuring activity when different substrate preparations are employed (Neverova et al., 1994). Furthermore, in some studies a mixture of truncated labeled oligosaccharides, Glc₃Man₁(7,9)GlcNAc, have been used (Taylor et al. 1994). This also results in erroneous measurement of activity since α-glucosidase I exhibits different affinity toward truncated triglucosylated oligosaccharide devoid of mannose residue(s), as discussed in section 1.8.3.

1.10.2 Spectrophotometric assay

The spectrophotometric assay uses a synthetic trisaccharide oligosaccharide, α-D-Glc-(1,2)-α-D-Glc-(1,3)-α-D-Glc-O-(CH₂)₈COOCH₃, which mimics the terminal glucotriose moiety of Glc₃Man₉GlcNAc₂ (Scaman et al. 1996). A spectrophotometric approach is used to determine the activity of α-glucosidase I based on the amount of glucose released from the synthetic substrate (Neverova et al. 1994). In this method, the amount of released D-glucose is measured by a coupling enzyme assay. D-glucose produced by α-glucosidase I is oxidized by glucose oxidase to glucuronic acid and hydrogen peroxide. The latter product is used by peroxidase to convert colorless reduced O-dianisidine to the brown oxidized form (Figure 1.7). This method was successfully used to determine the Km of α-glucosidase I isolated from S. cerevisiae (Neverova et al. 1994). This method, unlike the previous approach, can provide reproducible results. It has been reported that this synthetic trisaccharide can be hydrolyzed by α-glucosidase I from yeast, plant and mammalian sources (Gillmor et al. 2002; Neverova et al. 1994; Palcic et al. 1999; Romero et al. 1997). This indicates that the trisaccharide has the structural features necessary for interacting with the active site of orthologs.

The limitations of this method are mainly associated with the coupling assay. For instance, the minimum measurable α-glucosidase I activity is limited to the detection level of
the coupling assay. This would be a problem when it is required to measure very low α-
glucosidase I activity. Also any condition that can interfere with the coupling reaction would
affect glucosidase I activity measurement. As well, this method like the previous method
suffers from overestimation of α-glucosidase I activity when there are any impurities with α-
(1,3)- glucosidase activity (e.g. α-glucosidase II).
Figure 1.7. Illustration of the spectrophotometric assay of α-glucosidase I, adapted from Neverova et al. (1994).
1.11 MECHANISM OF $\alpha$-GLUCOSIDASE I

It is well established that hydrolysis of the glycosidic bond by glycosidases takes place through general acid-base catalysis that requires two amino acid residues where one serves as a proton donor and the other as a nucleophile/base. The hydrolysis occurs via two major mechanisms giving rise to either an overall retention or an inversion of configuration (Koshland 1953). The active site of both classes of enzyme generally contains a pair of carboxylic acid residues controlling the mechanism of action (Rye & Withers 2000). The distance between the catalytic residues is approximately 5.5Å for retaining glycosidase and 10Å for inverting enzymes (Rye & Withers 1999). In retaining glycosidases, cleavage of the glycosidic bond takes place through a double displacement mechanism. In the first step of this mechanism the carboxylic acid residue, which serves as the general acid, assists the cleavage of the glycosidic bond by protonating the glycosidic oxygen. In the next step the carboxylate side-chain deprotonates the incoming water molecule which then attacks the anomeric center and displaces the sugar (Figure 1.8) (Withers 2001).

Inverting glycosidases use the direct/single displacement mechanism through an oxocarbenium ion-like transition state. In this mechanism, one of the carboxylic acid residues provides the base catalytic assistance to attack the water while the other provides acid catalytic assistance for cleavage of the glycosidic bond (Figure 1.8) (Withers 2001).

NMR spectroscopic analysis of yeast and mammalian processing $\alpha$-glucosidase I using synthetic trisaccharide revealed that hydrolysis of the substrate takes place with a net inversion of configuration (Palcic et al. 1999).
Figure 1.8. Mechanism of inverting and retaining glycosidases, adapted from Withers (2001).
1.11.1 Inverting glycosidases

Elucidating the mechanism of action of a glycosidase is one of the most important steps towards understanding the specific function of an enzyme. Such mechanistic information is an essential component of inhibitor design, especially for those glycosidases that are associated with a metabolic disorder or a disease.

Mechanistic information is usually deduced from kinetic studies, stereochemistry studies, and three-dimensional structural (in free form and in ligand-complexed form) analysis of the wild-type as well as mutated variants. However, since within a family of glycoside hydrolases (URL: http://afmb.cnrs-mrs.fr/CAZY/) amino acid sequences and structures are assumed to be conserved it is also possible to infer important mechanistic information from a well-characterized member and apply it to other members (Henrissat 1991; Henrissat & Davies 1997). Also, some glycoside hydrolase families adopt similar folds and are therefore assigned into so-called “clans” (Table 1.1) (Zechel & Withers 2000). Thus, it is also plausible to retrieve imperative structural and mechanistic information from one well-studied member of a clan and expand it to others.

So far, over 25 families of glycoside hydrolases have been found to hydrolyze glycosidic bonds with a net inversion of configuration (URL: http://afmb.cnrs-mrs.fr/CAZY/), including processing α-glucosidase I (Palcic et al. 1999) of family 63 (clan GH-G, Table 1.1). However, structural information is not available for family 63 at the time of this study. In addition, the structure of family 37 (trehalase, EC 3.2.1.28) another member of clan GH-G (Table 1.1), has not yet been reported (URL: http://afmb.cnrs-mrs.fr/CAZY/). It is also noteworthy to point out that the processing α-glucosidase I (E.C. 3.2.1.106) possesses a unique α-(1,2)-D-glucan glucohydrolase activity. Collectively, these limitations
preclude inferring important mechanistic and structural information from other families of glycosyl hydrolases.

The mechanisms of inverting glycosidases are comparable in general therefore, glucoamylase (α-(1,4)-D-glucan glucohydrolase, EC 3.2.1.3), a well characterized α-inverting glycosidase, is discussed as a model. The majority of information available for glucoamylase has been obtained from *Aspergillus* sp. Glucoamylase is a member of family 15 (URL: http://afmb.cnrs-mrs.fr/CAZY/) and is assigned to the clan GH-L (Table 1.1). This enzyme exohydrolyzes α-(1,4)-glucose from the non-reducing ends of starch, and related oligo- and polysaccharides to produce β-D-glucose. This enzyme is able to cleave both α-(1,4)- and α-(1,6)-glucosidic linkages of starch at the same catalytic site, but it has been shown that cleavage of the latter bond is 500-fold slower than the former (Sierks et al. 1989). It has been found that glucoamylase from *Aspergillus* sp. (e.g. *A. awamori* var. *X100* and *A. niger*) comprises three domains; a starch-binding domain, a catalytic domain, and an O-glycosylated linker region in between (Sauer et al. 2000). The three dimensional structure of the catalytic domain (region 1-471) of glucoamylase (616 residues) from *A. awamori* var. *X100* contains 13 α-helices of which 12 form an (α/α)6-barrel (Figure 1.9) (Aleshin et al. 1992).

Site-specific chemical modification of carboxylic acid residues of glucoamylase with a water soluble carbodiimide suggested that Asp176, Glu179, and Glu 180 were likely important for the mechanism of action of the enzyme (Svensson et al. 1990). Site-directed mutagenesis studies have also attributed a role as a general acid to Glu179 (Sierks et al. 1990). It was reported that mutation of Glu179 to an isosteric Gln has resulted in undetectable activity with maltose and isomaltose (Sierks et al. 1990). The assignment of Glu179 as the general acid has been confirmed from three-dimensional observation of
glucoamylase structure in a complex with DNM (Harris et al. 1993). This finding has been further confirmed through structural analysis of the enzyme in a complex with acarbose, a tetrasaccharide inhibitor (Aleshin et al. 1994a).

Three-dimensional structural analysis of the catalytic domain of glucoamylase complexed with DNM, has revealed that the active site of the enzyme accommodates a single water molecule (Harris et al. 1993). This water molecule was observed at an approximate distance of 3.3 Å from Glu400, and has hydrogen bonding with the Glu400 and 6-OH of DNM. Therefore, it was suggested that the water molecule may serve as the nucleophile of a general base-catalyzed mechanism and that Glu400 may act as a general base catalytic residue (Harris et al. 1993). The isosteric mutation of Glu400 to Gln resulted in a change to the pH activity profile (Frandsen et al. 1994). Glu400Gln exhibits an elevated activity below pH 4 (the optimum pH for the wild-type enzyme), suggesting that only a single titratable carboxylic acid residue corresponding to Glu179 (the general acid) exists. The kcat values of Glu400Gln with maltose and maltoheptose were 35- and 60-fold lower, respectively, compared to wild type enzyme. The Km values of Glu400Gln towards maltose and isomaltose were increased 12- and 13-fold, respectively, resulting in a 200- to 400-fold reduction in kcat/Km. This is a relatively small loss in catalytic efficiency for the mutation of a key catalytic residue suggesting that other neighboring residues may have assumed the function of orienting and deprotonating the water molecule in the active site of the Glu400Gln mutant (Frandsen et al. 1994).

Carboxylic acid residues other than Glu179 and Glu400 have also been suggested to be critical for the mechanism of action of glucoamylase. Glu180 for instance, has been identified by kinetic studies and structural analysis as important for establishing a critical hydrogen bond with the 2-hydroxyl of the sugar in the +1 subsite, which is important for
stabilization of the transition-state (Frandsen et al. 1994; Stoffer et al. 1995). Kinetic studies and structure analysis have also suggested an important role for Asp55 in enzyme activity. Chemical modification of the carboxyl residues with carbodiimide showed that Asp55 has been modified (Svensson et al. 1990). Sierks and Svensson (1993) constructed a glucoamylase mutant, Asp55Gly, the non-conservative substitution of the carboxylic acid with Gly was to prevent any hydrogen bonding that may have occurred with Asp55 side chain. The kcat value for Asp55Gly was decreased approximately 200-fold with maltose and isomaltose. The Km values remained unchanged comparing to the wild-type enzyme. Collectively, this led these workers to propose that this residue has a role in transition-state stabilization (Sierks & Svensson 1993). Kinetic studies and structural analysis together, revealed that this residue is important for establishing interactions with the 4'- and 6'-hydroxyl groups of the glucose in the -1 subsite (non-reducing), suggesting it is important in the transition-state (Aleshin et al. 1994a; Aleshin et al. 1994b; Sierks & Svensson 1993). Based on kinetic studies it is proposed that interactions of the enzyme with the 4'- and 6'-hydroxyl of the glucosyl group in subsite -1 is important for the substrate binding while interactions with subsite +1 may be essential for the product release (Sierks & Svensson 2000).

Mutation of general base (Glu400) to Cys and oxidizing the Cys400 with iodoacetic acid resulted in formation of cysteinesulphinic acid (Cys-SO2H) (Fierobe et al. 1998). This modification was concomitant with elevating activity, 700-fold increase in kcat value, and an increased distance between the two catalytic carboxylic acid residues (Glu179 and Cys400-SO2H). Therefore, it was suggested that the distance between catalytic carboxylic acid residues, which is generally considered critical for the nucleophile attack on the glycosidic
bond and that the chemical nature of catalytic base, is not very restricted for this inverting enzyme (Fierobe et al. 1998).

Table 1.1. Classification of glycoside hydrolases into different clans based on fold similarity*.

<table>
<thead>
<tr>
<th>Clan</th>
<th>Fold</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH-A</td>
<td>(β/α)8</td>
<td>1 2 5 10 17 26 30 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39 42 50 51 53 59 72 79</td>
</tr>
<tr>
<td>GH-B</td>
<td>β-jelly roll</td>
<td>7 16</td>
</tr>
<tr>
<td>GH-C</td>
<td>β-jelly roll</td>
<td>11 12</td>
</tr>
<tr>
<td>GH-D</td>
<td>(β/α)8</td>
<td>27 36</td>
</tr>
<tr>
<td>GH-E</td>
<td>6-fold β-propeller</td>
<td>33 34 83</td>
</tr>
<tr>
<td>GH-F</td>
<td>5-fold β-propeller</td>
<td>43 62</td>
</tr>
<tr>
<td>GH-G</td>
<td>-</td>
<td>37 63</td>
</tr>
<tr>
<td>GH-H</td>
<td>(β/α)8</td>
<td>13 70 77</td>
</tr>
<tr>
<td>GH-I</td>
<td>α+β</td>
<td>24 46 80</td>
</tr>
<tr>
<td>GH-J</td>
<td>5-fold β-propeller</td>
<td>32 68</td>
</tr>
<tr>
<td>GH-K</td>
<td>(β/α)8</td>
<td>18 20</td>
</tr>
<tr>
<td>GH-L</td>
<td>(α/α)6</td>
<td>15 65</td>
</tr>
<tr>
<td>GH-M</td>
<td>(α/α)6</td>
<td>8 48</td>
</tr>
<tr>
<td>GH-N</td>
<td>(β)-helix</td>
<td>28 49</td>
</tr>
</tbody>
</table>

* Information acquired from CAZy server (URL: http://afmb.cnrs-mrs.fr/CAZY/).
Figure 1.9. Schematic of three-dimensional structure of *A. awamori* X100 glucoamylase (catalytic domain) in a complex with DNM (Protein Data Bank: 1dog). Image viewed from the top. α-Helices (labeled with α(1-13)) render into an (α/α)6 barrel fold (Aleshin et al. 1992). Atoms of the glycosyl chains are represented by open circles (surrounding the molecule). Two molecules of DNM (centre of illustration) were found to occupy in the complex with the catalytic domain (Harris et al. 1993). The illustration was produced by SwissPdb Viewer V 3.7 (http://www.expasy.org/spdbv/) (Guex & Peitsch 1997).
1.12 PURIFICATION OF α-GLUCOSIDASE I

For detailed biochemical and kinetic studies, it is necessary to have pure α-glucosidase I. Any contamination with other glycosidases, such as α-(1,3)-glucosidase, can confound the results especially in kinetic studies. In this regard, several procedures have been described for purifying α-glucosidase I from different eukaryotic cells (Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). In all of these studies α-glucosidase I, which is predominantly associated with the membrane, was isolated from the microsomes by solubilizing the activity with a buffer containing a low percentage (0.1-0.8%) of detergent such as Triton X-100 or Lubrol PX. Subsequently, solubilized activity was purified by applying a series of ion exchange and affinity chromatographies (i.e. DNM and ConA). These methods purified α-glucosidase I from lower and higher eukaryotes with different yields, varying from 6 to 20% (Bause et al. 1986; Bause et al. 1989; Shailubhai et al. 1987; Zeng & Elbein 1998). The highest purification yield (55%) was reported for α-glucosidase I from calf liver with single step chromatography on a DNM-based resin (Hettkamp et al. 1984).

Affinity ligands based on DNM and DNM-alkylated derivatives have been used widely for the purification of α-glucosidase I. Synthesis of DNM-based resins by coupling the alkylated derivative of DNM, such as N-5'-CP-DNM (CP-DNM) and N-methyl-N-5'-CP-DNM, to the base resin through the carbodiimide procedure have been described in several reports (Bause et al. 1991; Bernotas & Ganem 1990; Hettkamp et al. 1984; Sehgal & Vijay 1994). However it was reported that purification of α-glucosidase I by DNM-based resin required prolonged (i.e. overnight) batch-wise mixing with enzyme at 4°C for effective binding of glucosidase I activity (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). α-Glucosidase I tightly bound, to the DNM-
based resin, was commonly eluted by a buffer containing DNM (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987). Alternatively Zeng and Elbein (1998) reported successful elution of the mung bean α-glucosidase I bound to the DNM resin using 100 mM trehalose (α-D-Glc-(1,1)-α-D-Glc).

ConA-based affinity chromatography has also been used for purification of α-glucosidase I (Bause et al. 1986; Zeng & Elbein 1998). ConA is a lectin protein isolated from jack bean with high affinity for the trimannoside, 3,6-di-O-(α-D-mannopyranosyl)-D-mannose, attached to GlcNAc (Figure 1.2), which is present in all N-glycan types (Chervenak & Toone 1995; Dam et al. 1998). ConA-based affinity chromatography improved purity of α-glucosidase I from mung bean and S. cerevisiae when applied in conjunction with DNM-based chromatography. The plant and yeast α-glucosidase I bound to ConA was eluted by a buffer containing 0.4 M and 1 M methyl-α-D-mannoside, respectively (Bause et al. 1986; Zeng & Elbein 1998). Bause et al. (1989) reported that α-glucosidase I from pig liver bound tightly to ConA-Sepharose resin, yet when enzyme was applied in a buffer containing 0.8 M methyl-α-D-mannoside, binding was prevented. However, it was noted that bound α-glucosidase I could not be eluted from the ConA. Therefore, it was suggested that conformational changes in oligosaccharide/protein structure likely enhanced pig liver α-glucosidase I binding to ConA and hindered dissociation (Bause et al. 1989).

It has become evident from the purification studies that, regardless of the source of α-glucosidase I, a significant increase in purity (ca 160-960 fold) is achieved when DNM-based affinity chromatography is employed (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). The major drawback associated with this step was the relatively low yield of activity. The lowest yield (18%) was reported for bovine mammary gland enzyme (Shailubhai et al. 1987), while higher yields were
reported for α-glucosidase I from the mung bean (39%) and *S. cerevisiae* (60%) (Bause et al. 1986; Zeng & Elbein 1998). However, the low recovery could be associated with overestimating α-glucosidase I activity before this stage that contained α-glucosidase II. In several studies, it has been reported that DNM-based affinity chromatography alone could not provide homogeneous α-glucosidase I suggesting further purification was required (Bause et al. 1986; Bause et al. 1989; Shailubhai et al. 1987; Zeng & Elbein 1998). Furthermore, it should be noted that employing DNM based affinity resins as the predominant step for purification of α-glucosidase I is dependent on the enzyme activity. Therefore, when purification of the inactive form of α-glucosidase I (i.e. α-glucosidase I mutants) is required, this method needs to be replaced by an alternative approach independent from activity.

Molecular mass of the subunit of purified α-glucosidase I orthologs has been determined by SDS-PAGE. Subunit masses of 95, 97, and 85 kDa were reported for *S. cerevisiae*, mung bean, and bovine α-glucosidase I, respectively (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). Molecular mass of recombinant human hippocampus α-glucosidase I subunit isolated from the transfected mammalian cells was determined to be 95 kDa (Kalz-Fuller et al. 1995). However, the molecular mass of the native form of α-glucosidase I from different bovine tissues (i.e. mammary and liver) was determined to be approximately 320-350 kDa by gel-filtration (Sephacryl S-300) suggesting that the native form of the mammalian enzyme is comprised of four identical subunits with the molecular mass of 85 kDa (Hettkamp et al. 1984; Shailubhai et al. 1987). However, it is not yet known if the enzyme from yeast and plant is also an oligomeric protein.
1.12.1 Partial purification of the yeast soluble $\alpha$-glucosidase I

Although it has been established that $\alpha$-glucosidase I is predominantly associated with the membrane of the ER, an unbound (soluble) form of activity has been isolated from *S. cerevisiae*. Kilker et al. (1981) reported that approximately 50% of the total $\alpha$-(1,2)glucosidase activity was detected in the supernatant of the cell free extract. They also reported that ultra-centrifugation of the crude extract containing microsomes did not sediment the soluble activity and that using detergent (Triton X-100) only stimulated activity associated with the membrane (~45%) without any effect on the soluble form of activity. It was also shown that the soluble form of yeast $\alpha$-glucosidase I can be partially purified by a combination of ammonium sulfate precipitation and anion exchange chromatography with no apparent aryl $\alpha$-glucosidase activity (Kilker, Jr. et al. 1981). Partially purified enzyme preparations showed low activity toward Glc$_2$Man$_3$GlcNAc$_2$, the $\alpha$-glucosidase II substrate, indicating that the preparation was not free from $\alpha$-(1,3)-glucosidase activity. Further studies revealed that the $K_m$ values of the soluble and membrane forms of $\alpha$-glucosidase I, using synthetic glucotriose substrate, are different. $K_m$ values of 1.28 and 0.68 mM were reported for the soluble form and detergent-extracted enzyme, respectively (Neverova et al. 1994). It was also reported that DNM inhibits the soluble form of $\alpha$-glucosidase I activity with a $K_i$ value of 50 $\mu$M (Neverova et al. 1994). Although it was not experimentally established that the soluble form of $\alpha$-(1,2) glucosidase activity is derived from the membrane-bound enzyme, it was speculated that the soluble form of yeast $\alpha$-glucosidase I was a proteolytic product of the membrane form (Herscovics 1999b).
1.13 CATALYTIC DOMAIN OF $\alpha$-GLUCOSIDASE I

Bause and co-workers (1989) used trypsin at room temperature to hydrolyze pure native $\alpha$-glucosidase I from pig liver, with a molecular subunit mass of 85 kDa. After 40 min of hydrolysis, three major polypeptides with masses of 69, 45, and 29 kDa were produced in which the 29 kDa polypeptide was the predominant fragment. Interestingly, almost 60% of the total initial activity was retained after 40 min of hydrolysis indicating that the catalytic domain of $\alpha$-glucosidase I is resistant to proteolysis (Bause et al. 1989). However, they were not able to separate any active polypeptide from the mixture of polypeptide fragments using different techniques such as high resolution gel chromatography. Furthermore, deglycosylation of the polypeptides with endo H revealed that the 69 and 45 kDa fragments and not the 29 kDa polypeptide were $N$-glycosylated (Bause et al. 1989). Further studies by Shailubhai et al. (1991) showed that controlled digestion of rat mammary $\alpha$-glucosidase I (85 kDa) by trypsin at 37°C for 60 min produced a mixture of polypeptides in which the 39 kDa polypeptide was the dominant fragment. It was also reported that during the first 15 min of digestion, polypeptides with higher molecular masses, 69 and 55 kDa, were generated. It was noted that approximately 60% of the initial total activity was retained after 60 min of digestion. The 39 kDa active polypeptide was isolated from the digested mixture of peptide fragments using a CP-DNM affinity resin, indicating that the fragment contained a putative catalytic domain. In addition, digestion of saponin-permeabilized membrane by trypsin released the soluble 39 kDa fragment while the 69 and 55 kDa fragments remained associated with the membrane suggesting the catalytic domain was oriented toward the lumen of the ER. Subsequently it was reported that $\alpha$-glucosidase I from several different tissues of rat, mouse, guinea pig and bovine mammary glands, sheep liver, and pig kidney had an identical molecular subunit mass of 85 kDa (Pukazhenthi et al. 1993b). Hydrolysis
of these α-glucosidase I orthologs for 90 min at 37°C with trypsin resulted in formation of several polypeptides with different molecular masses which varied from 29 kDa to 69 kDa (Pukazhenthi et al. 1993b). This indicated that the mammalian α-glucosidase I orthologs contained regions resistant to trypsin hydrolysis (Pukazhenthi et al. 1993b).

Recently Romaniouk et al. (2004) developed a photo-affinity labeling approach to identify the catalytic region of α-glucosidase I. They synthesized a photoactive derivative of DNM, 4-ρ-azidosalicylamido-butyl-5-amido-pentyl-1-DNM, to probe near or at the binding site of α-glucosidase I. The photo-labeling of native bovine mammary α-glucosidase I was followed by digestion of the labeled enzyme with V8-protease (from Staphylococcus aureus) which resulted in a 24 kDa photo-labeled polypeptide, suggesting that this was the catalytic domain (Romaniouk et al. 2004). In addition, they developed a mono-specific antibody against the highly conserved region of mammalian α-glucosidase I, DDYPRASHPSTTERHL (Romaniouk et al. 2004), which contained part of the suggested mammalian binding motif (ERHDLRCW) (Romaniouk & Vijay 1997). Western blot analysis showed the 24 kDa polypeptide generated by V8-protease hydrolysis of non-labeled α-glucosidase I was recognized by that mono-specific antibody, indicating that this polypeptide contained the putative binding motif (Romaniouk et al. 2004).

The exact location of these active polypeptides isolated from mammalian α-glucosidase I has not yet been reported. Furthermore, it is not understood if the catalytic domain is resistant to proteolysis and can be isolated from other α-glucosidase I orthologs such as plant and yeast α-glucosidase I. Investigation about the catalytic region of plant and yeast enzyme could provide better insight about structural conservation and similarity between orthologs.
1.14 CRITICAL RESIDUES OF PROCESSING α-GLUCOSIDASE I

1.14.1 Potential binding site residues

Specific chemical modification of Arg, Cys, Trp, and Tyr in rat α-glucosidase I resulted in almost abolished activity while His modification only partially (29%) reduced enzyme activity (Romaniouk & Vijay 1997). Also, DNM was able to protect rat α-glucosidase I from inactivation against the chemical modifications of Arg, Cys and Trp, but not Tyr (Pukazhenthi et al. 1993a; Romaniouk & Vijay 1997). As well, the 39 kDa active polypeptide isolated by tryptic digestion from rat α-glucosidase I was specifically labeled by a sulfhydryl group chemical reagent, thereby showing participation of the critical Cys residue in the putative catalytic domain of mammalian α-glucosidase I (Pukazhenthi et al. 1993a).

Based on these studies it was suggested that Arg, Cys, and Trp likely play crucial roles as the binding residues of the mammalian enzyme (Romaniouk & Vijay 1997). Therefore, based on these chemical modifications and studies which showed that the catalytic domain of rat α-glucosidase I, was a 39 kDa glycopeptide located at the C-terminus, it was suggested that the 591ERHLDLRCW600 region in rat α-glucosidase I likely serves as the binding motif (Romaniouk & Vijay 1997). This motif is adjacent to the only predicted N-glycosylation site of the enzyme, Asn654. By contrast, it was reported that α-glucosidase I from mung bean was sensitive to His modification and not Cys (Zeng & Elbein 1998). However, it has not yet been determined if the His modification could be prevented by protection of active site with DNM or any other inhibitors. As well it is not yet known if the suggested residues are conserved between mammalian and lower eukaryotes such as yeast.
1.14.2 Other structural and functional residues

In addition to the above reported potential binding residues, recent studies on cell mutants with an α-glucosidase I deficiency revealed other conserved residues from different orthologs that may play important structural or functional roles. It was reported that an infant suffering from CDG type-IIb disorder had mutations at Arg486Thr and Phe652Leu that drastically reduced α-glucosidase I activity (~99%) (Volker et al. 2002). Also, recombinant mutated enzymes (Arg486Thr and Phe652Leu) were expressed in transfected mammalian cells without any detectable α-glucosidase I activity. However, it was shown that Arg486Thr and not Phe652Leu could partially bind to CP-DNM resin and be eluted by DNM, indicating that Arg486 and Phe652 may have different functions regarding protein folding and formation of the active site (Volker et al. 2002).

It has also been demonstrated that Lec23 CHO cells, isolated from a mutagenized population resistant to lectin, do not possess α-glucosidase I activity (Ray et al. 1991). Subsequently it was determined that α-glucosidase I inactivation in Lec23 cells was due to a single mutation at Ser321 to Phe (Hong et al. 2004). The same positional mutation (Ser440Phe) in a recombinant human α-glucosidase I abolished ~98% of the activity (Hong et al. 2004). In *S. cerevisiae*, mutation at Gly725Arg of α-glucosidase I is reported to impair α-glucosidase I activity (Hitt & Wolf 2004). Although all of these mutations took place at highly conserved residues within the C-terminus domain of the enzyme where the putative catalytic domain is located, there is no available kinetic or structural analyses to indicate if these mutations play a direct function in catalysis or have structural implications.
1.15 IMPORTANCE OF YEAST IN N-GLYOSYLATION STUDIES

*S. cerevisiae*, without doubt, is one of the most prominent and useful model organisms for glycosylation studies because it easily adapted to genetic and molecular techniques and its genome has been completely sequenced (Cherry et al. 1997). Furthermore, many of the steps in the yeast N-glycosylation pathway have a high degree of conservation with higher eukaryotes, including oligosaccharide lipid synthesis, glycan transfer to nascent proteins by OST, and N-glycan processing in the ER (Byrd et al. 1982; Trimble et al. 1980). Indeed, many of the pathways and enzymes involved in the early N-glycosylation have been found in mammals by homology with their yeast counterparts (Gemmill & Trimble 1999). In addition, it seems *S. cerevisiae* is not highly sensitive to the modifications involving N-glycan processing as mutated yeast strains with impaired processing glycosidases (i.e. α-glucosidase I and II) were viable and grew normally with no major impact on maturation of the majority of the N-glycans (Esmon et al. 1984; Jakob et al. 1998b).

1.15.1 CWH41 encodes α-glucosidase I

The cwh41 mutant was first isolated during screening of yeast cell wall mutants which exhibited calcofluor white hypersensitivity (*cwh*) (Ram et al. 1994). Jiang et al. (1996) successfully cloned *CWH41* in *S. cerevisiae*. Expressed Cwh41p was localized in the ER and it was also observed that transformed yeast treated with tunicamycin, an inhibitor of N-glycosylation, produced Cwh41p with a smaller molecular mass suggesting Cwh41p is N-glycosylated (Jiang et al. 1996). However, these workers were not able to identify Cwh41p as α-glucosidase I and suggested that Cwh41p was a type II membrane protein likely playing a functional role in the biosynthesis of cell wall β-1,6-glucan (Jiang et al. 1996). Further
studies demonstrated that the role of \textit{CWH41} in biosynthesis of \(\beta\)-1,6-glucan is indirect (Abeijon & Chen 1998). Romero et al. (1997) demonstrated that yeast cwh41\(\Delta\) mutants lack any detectable \(\alpha\)-glucosidase I activity indicating that \textit{CWH41} (YGL027C) encodes processing \(\alpha\)-glucosidase I. \textit{CWH41} (GenBank: U35669) is located on chromosome VII and is 2.5 kb in length, encoding 833 amino acid residues. Cwh41p has 24 and 20% overall identity with human and \textit{Arabidopsis} \(\alpha\)-glucosidase I, respectively (Gillmor et al. 2002; Romero et al. 1997).

Studies with \(\alpha\)-glucosidase I face several challenges which consequently hinder further characterization and mechanistic investigations, including difficulties in acquiring sufficient quantity of a homogeneous preparation of \(\alpha\)-glucosidase I, instability of purified enzyme, and substrate limitations. It was reported that expression of recombinant human \(\alpha\)-glucosidase I in transfected cells was concomitant with only a 4-fold increase of activity (Kalz-Fuller et al. 1995). Cloning of cDNA encoding mouse \(\alpha\)-glucosidase did not result in expression of significant quantities of \(\alpha\)-glucosidase I activity unless it was co-transfected with cDNAs of chaperones calnexin and calreticulin, and ERp57 (Romaniouk et al. 2004). These results clearly indicate that production of the recombinant mammalian \(\alpha\)-glucosidase I at a level that can be used for further structural-kinetic characterization, and especially crystallization, is not promising at the current time. Moreover, it was reported that the purified native form of \(\alpha\)-glucosidase I from mammalian cells had a half life of 1-3 weeks at 2-4\(^\circ\)C suggesting either that the preparation was not homogeneous or that mammalian \(\alpha\)-glucosidase I is rather unstable (Hettkamp et al. 1984; Shailubhai et al. 1987). Therefore, using the yeast enzyme can likely overcome the current existing problems by employing large scale production of recombinant enzyme and establishing a robust purification
procedure. Studies on yeast α-glucosidase I will ultimately provide a better understanding about family 63 glycoside hydrolases.
OVERALL HYPOTHESES

The soluble α-glucosidase I from *S. cerevisiae* possesses comparable structural and biochemical characteristics to other α-glucosidase I orthologs and can be used as a model for the investigation of family 63 of glycosyl hydrolases.

OVERALL OBJECTIVES

To establish a robust purification procedure; to identify functional residues (i.e. potential binding and catalytic residues); to investigate domains of yeast α-glucosidase I.

PART 1. PARTIAL PURIFICATION AND CHARACTERIZATION OF THE SOLUBLE FORM OF PROCESSING α-GLUCOSIDASE I.

**Hypothesis:** The soluble form of *S. cerevisiae* α-glucosidase I is the hydrolytic fraction of the membrane-bound α-glucosidase I, which is encoded by CWH41.

**Objective 1:** To establish a protocol which is comprised of a series of ion-exchange and affinity chromatographies for the purification of the soluble processing α-glucosidase I from *S. cerevisiae*.

**Objective 2:** To determine whether His, Cys and carboxylic acid residues are important in yeast α-glucosidase I activity and to establish if the soluble α-glucosidase I is glycosylated.

PART 2. PURIFICATION OF THE SOLUBLE α-GLUCOSIDASE I TO HOMOGENEITY.
**Hypothesis 1:** Expression level and purification of the soluble α-glucosidase I from the recombinant yeast can be improved using a fermenter and DNM-based affinity chromatography.

**Objective 1:** To investigate if the production, purification yield, and purity level of the soluble α-glucosidase I can be improved using a fermenter and employing an DNM-based affinity chromatography.

**Hypothesis 2:** Catalytic region of yeast α-glucosidase I like its mammalian counterpart is resistant to hydrolysis and can be liberated as a catalytically active fragment by proteolysis.

**Objective 2:** To evaluate if endogenous hydrolysis of partially purified α-glucosidase I and proteolysis of pure α-glucosidase I can generate any proteolytic resistant fragments which exhibit glucosidase I activity.

**PART 3. CATALYTIC DOMAIN AND BINDING RESIDUES OF α-GLUCOSIDASE I.**

**Hypothesis 1:** Potential binding residues have been conserved between mammalian and yeast α-glucosidase I.

**Objective 1:** To identify the potential binding residues of the yeast α-glucosidase I using site specific chemical modification.

**Hypothesis 2:** The 37 kDa polypeptide generated by endogenous and/or trypsin hydrolysis contains the putative catalytic domain.

**Objective 2:** To generate, isolate, and characterize the 37 kDa catalytically active fragment.
PART 4. FUNCTIONAL DOMAINS AND IMPORTANT CARBOXYLIC ACID RESIDUES (E613 and D617) OF α-GLUCOSIDASE I.

**Hypothesis 1**: Truncated forms of Cwh41p containing putative catalytic domains can be expressed as catalytically active proteins.

**Objective 1**: To express, purify, and characterize truncated forms of Cwh41p containing the putative catalytic domain.

**Hypothesis 2**: Highly conserved Glu613 and Asp617 residing in the putative catalytic region play functional roles in α-glucosidase I activity.

**Objective 2**: To examine the importance of Glu613 and Asp617 in enzyme activity using site-directed mutagenesis.
CHAPTER II

PART 1. PARTIAL PURIFICATION AND CHARACTERIZATION OF

THE SOLUBLE FORM OF PROCESSING α-GLUCOSIDASE I
2 CHAPTER II

Preface

A version of Chapter II has been published in Glycobiology 2002.


* Authors contributed equally
2.1 ABSTRACT

Processing α-glucosidase I is an ER membrane protein that hydrolyzes the α-(1,2) glucose residue from the oligosaccharide Glc₃Man₂GlcNAc₂ and may play an important role in the quality control of glycoprotein biosynthesis in the endoplasmic reticulum. It was reported that CWH41 encodes this membrane-bound enzyme (Cwh41p) in S. cerevisiae. The soluble α-glucosidase I was isolated from transformed S. cerevisiae overexpressing CWH41. The ratio of the soluble α-glucosidase I to the membrane form was increased 9-fold in comparison to wild type yeast reported by Kilker et al. (1981) suggesting the soluble enzyme is encoded by CWH41. The soluble form of activity was purified to 95% homogeneity using a combination of ammonium sulfate precipitation, anion-exchange, lectin affinity, and size-exclusion chromatographies. The molecular subunit mass of the soluble α-glucosidase I was determined to be 98 kDa by SDS-PAGE. N-terminal sequencing of the soluble enzyme revealed that it results from cleavage between residues Ala24 and Thr25 of the transmembrane domain of Cwh41p. This cleavage could be partially inhibited by addition of leupeptin and pepstatin during enzyme isolation. Binding of the soluble α-glucosidase I to ConA and decrease in the mass of enzyme, ~ 5 kDa, after treatment by N-glycosidase F indicated that the soluble enzyme is N-glycosylated. The soluble α-glucosidase I was sensitive to modification of His and not Cys, suggesting that mechanistically it is more similar to the plant than mammalian form of the enzyme. Site-specific chemical modification of the soluble α-glucosidase I from yeast using 3-(3-(dimethylamino)propyl)carbodiimide revealed that there is a catalytically active carboxylic acid residue (i.e. Asp/Glu) susceptible to modification, as this residue could be protected using the inhibitor deoxynojirimycin.
2.2 INTRODUCTION

Processing α-glucosidase I or mannosyl-oligosaccharide glucosidase (EC 3.2.1.106) is a key enzyme in the biosynthesis of N-glycoproteins in the ER. α-Glucosidase I commences trimming of N-glycans by exo-hydrolysis of the α-(1,2)-linked glucose unit from the newly assembled Glc₃Man₉GlcNAc₂ after en bloc transfer to the nascent sequence (Asn-X-Thr/Ser) of proteins (Grinna & Robbins 1980). In the N-glycosylation pathway, α-glucosidase I is situated at the critical position which suggests that it likely regulates the level of Glc₃Man₉GlcNAc₂ linked to its lipid carrier and participates in glycoprotein quality control (QC) (Helenius & Aebi 2004; Spiro & Spiro 1991). The action of α-glucosidase I is essential for further ER processing steps which are controlled by α-glucosidase II and α-mannosidase, respectively (Kornfeld & Kornfeld 1985). Folding of monoglucosylated N-glycoproteins, the product of the α-glucosidase II, is promoted by interaction with lectin chaperones (Ware et al. 1995). However, lectin chaperones are unable to recognize tri- and diglucosylated N-glycoproteins (Spiro et al. 1996). Thus impairing α-glucosidase I activity interferes with glycoprotein folding and QC. While a minor pathway administrated by a Golgi endomannosidase in some eukaryotes can by-pass the blocked α-glucosidase I (Moore & Spiro 1990), endomannosidase can not completely compensate for the α-glucosidase I. In vivo studies have indicated that function of α-glucosidase I is vital for normal plant and human development and viability (Boisson et al. 2001; De Praeter et al. 2000).

Despite the importance of α-glucosidase I, there is a limited understanding of the structure, characteristics, and mechanism of α-glucosidase I due to substrate specificities, instability, and difficulties in obtaining sufficient amount of homogeneous enzyme. This enzyme has been placed in family 63 of glycosyl hydrolases with no homology with other
known families (URL: http://afmb.cnrs-mrs.fr/CAZY/) which also hinders obtaining more information about this enzyme.

α-Glucosidase I from lower and higher eukaryotes exhibit comparable biochemical characteristics such as the activity toward the natural substrate (Glc₃Man₉GlcNAc₂) and near neutral pH optimum as well as sensitivity toward inhibitors such as DNM (Bause et al. 1986; Schweden et al. 1986; Zeng & Elbein 1998). Furthermore, yeast, mammalian, and plant α-glucosidase I share regions of significant amino acid identity (20-24%) suggesting the gene has been conserved during evolution (Gillmor et al. 2002; Romero et al. 1997). However, several discrepancies have been noted between mammalian and other α-glucosidase I orthologs. It was reported, for instance, that removing mannose residues from the natural oligosaccharide resulted in reducing mammalian α-glucosidase I activity while this stimulated the activity of plant and yeast enzyme (Grinna & Robbins 1980; Saunier et al. 1982; Zeng & Elbein 1998). Furthermore, mammalian enzyme is sensitive to chemical modification of Cys residues and not His while the opposite observation was reported for the plant enzyme (Zeng & Elbein 1998).

It is known that α-glucosidase I is isolated from the membrane of different eukaryotic cells, i.e. yeast, plant and mammalian (Bause et al. 1989; Hetttkamp et al. 1984; Zeng & Elbein 1998). However, yeast α-glucosidase I activity has also been isolated as a soluble form (Kilker, Jr. et al. 1981). As well, overexpression of CWH41, the gene encoding membrane-bound yeast α-glucosidase I (Romero et al. 1997), in S. cerevisiae was concomitant with 28-fold increase in the soluble activity (Dhanawansa et al. 2002) suggesting that the soluble form of α-glucosidase I likely is encoded by the same gene (CWH41). This study was focused on purifying the soluble form of S. cerevisiae α-glucosidase I and to determine if the soluble form of the enzyme is a proteolytic product of
the membrane enzyme. In addition, some characteristics of the soluble enzyme such as glycosylation and sensitivity to inhibitors were investigated and the sensitivity of the enzyme to modification of critical amino acid residues (i.e. Cys, His, and Asp/Glu) was examined.

2.3 MATERIALS AND METHODS

2.3.1 Materials

All chemicals used in this study were reagent grade and obtained from Sigma, except where noted. Synthetic trisaccharide was kindly provided by Dr. M. Palcic, Department of Chemistry, University of Alberta. Dialysis membrane used in this study had a molecular weight cut-off of 12,000-14,000 Da and was purchased from Fisher.

2.3.2 Yeast strain and growth media

Transformed *S. cerevisiae* overexpressing *CWH41* was used for this study. Transformation of *S. cerevisiae* was completed prior to this study as described by Dhanawansa et al. (2002). Open reading frame (ORF) of *CWH41* was amplified from the yeast episomal plasmid (YEp351), containing the ORF of *CWH41* (Jiang et al. 1996) and subcloned into a yeast shuttle vector pHVX2 containing phosphoglycerate kinase gene (*PGK1*) promoter (*PGK1p*) and terminator (*PGK1T*) sequences (Dhanawansa et al. 2002). The newly formed plasmid (pRAN1) was transformed in *S. cerevisiae* AH22 (*MATa, Leu2-3, leu2-112, his4-519, can1, [cir+]*, ATCC 38626) (Dhanawansa et al. 2002). Yeast transformants were grown in a minimal medium containing 0.17% yeast nitrogen base without amino acids (Difco), 0.5% ammonium sulfate, 2% glucose, and 50 mg/l L-histidine. Solid medium was prepared by adding 1.5% agar (Difco) to liquid medium. The stock culture was prepared by mixing an equal volume of the overnight grown yeast in an incubator
shaker (New Brunswick Scientific Co.), at 28°C and 300 rpm, with 30% (w/v) sterile glycerol (Fisher). Aliquots of 1.5 ml of the stock culture were dispersed in 2 ml of sterile cryogenic vials (Corning) and kept at -80°C freezer for the long term storage.

2.3.3 Growth of transformed yeast

Sterile glass tubes containing 2 ml of media were inoculated with the transformed *S. cerevisiae* from the plate and were grown overnight in a shaking incubator at 28°C and 300 rpm. The overnight 2 ml culture was used to inoculate 250 ml of fresh media in 1000 ml sterile flask and incubated at 28°C, with shaking at 300 rpm. The growth curve of the transformed yeast was determined by measuring turbidity of the culture at 1-h time intervals at 600 nm with a spectrophotometer (UNICAM UV/VIS Spectrometer UV2 ATI UNICAM).

2.3.4 Isolation of the soluble and membrane-bound α-glucosidase I

Biomass of transformed *S. cerevisiae* was harvested from 1-1.5 litre (4-6×250 ml) of the culture at the mid-log phase after 17 h incubation at 28°C, with shaking at 300 rpm. All isolation and purification steps of α-glucosidase I were carried out at 4°C. Cultures were centrifuged at 7,000 ×g for 15 min, the supernatant was discarded, and the weight of the biomass was measured. Thereafter, the packed biomass was washed with cold (4°C) 10 mM sodium phosphate buffer, pH 6.8 and centrifuged again. The cells pellet was resuspended in four volumes of the same phosphate buffer containing 5 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor. Approximately 15 ml of cell suspension was transferred to the 50 ml polypropylene centrifuge tubes and sterile acid-washed glass beads (ratio of biomass:beads, 1:4) were added. Cell disruption was completed by vortexing for 10 min with sterile acid-washed glass beads, with alternating 1-min intervals of vortexing and
cooling on ice. Cell disruption was checked by microscopic observation of the cells stained with aqueous methylene blue (0.1%). The cell homogenate was centrifuged at 16,000 \( \times \) g for 20 min to remove cell debris and glass beads. The supernatant was collected and the cell debris was mixed with the same buffer and centrifuged to isolate the remaining protein. This process was repeated two more times. The resultant supernatant was then centrifuged at 100,000 \( \times \) g for 60 min. The supernatant was used for the isolation of the soluble \( \alpha \)-glucosidase I and the microsomal pellet was used for the membrane-bound form of the enzyme.

### 2.3.5 Purification of the soluble \( \alpha \)-glucosidase I

The soluble protein fraction containing \( \alpha \)-glucosidase I activity was precipitated between 20-60% saturated ammonium sulfate and isolated by centrifugation at 15,000 \( \times \) g for 30 min, as described by Kilker et al (1981). The protein precipitate was dissolved in 10 mM sodium phosphate buffer, pH 6.8 containing 5 mM PMSF. Since it was reported that ammonium sulfate has an inhibitory effect on \( \alpha \)-glucosidase I (Kilker, Jr. et al. 1981), the protein solution was dialyzed for a minimum of 4 h against the same buffer to remove ammonium sulfate.

The theoretical isoelectric point (pI) of Cwh41p (Swiss-Prot: P53008) is calculated as 4.96 (URL: http://ca.expasy.org/cgi-bin/pi_tool1?P53008). The acidic pI indicates that Cwh41p at pH 6.8 would likely be negatively charged. Therefore anion-exchange chromatography was employed for the early step of purification. Using anion-exchange chromatography for partial purification of the soluble form of yeast \( \alpha \)-glucosidase I has been reported elsewhere (Kilker, Jr. et al. 1981; Neverova et al. 1994). The dialyzed sample from ammonium sulfate precipitation was applied to a Toyopearl DEAE column (2.5x20 cm).
equilibrated with 10 mM sodium phosphate buffer, pH 6.8, at a flow rate of 1.5 ml/min. Unbound proteins were eluted with two column volumes of 10 mM sodium phosphate buffer, pH 6.8. The column was then eluted stepwise with 0.1, 0.2, and 0.3 M NaCl in the loading buffer with a flow rate of 2-2.5 ml/min. The 0.2 M NaCl elution, containing α-glucosidase I activity, was dialyzed for 4 h in 10 mM sodium phosphate buffer, pH 6.8. The dialyzed sample was concentrated to approximately 10 ml using an Amicon 8050 Ultrafiltration cell with an Ultrace YM10 membrane (Millipore). The concentrated sample was then applied to a fast protein liquid chromatography (FPLC) system using an anion-exchange MonoQ HR 5/5 column (Amersham-Pharmacia Biotech) equilibrated with 10 mM sodium phosphate buffer, pH 6.8 (loading buffer). The flow rate was adjusted to 0.6 ml/min and fractions of 1 ml were collected. A continuous buffer gradient ranging from 0-1 M NaCl in loading buffer was used to elute proteins. Fractions with α-glucosidase I activity were pooled.

The active fraction (3-4 ml) from the MonoQ HR 5/5 was exchanged into a ConA loading buffer (10 mM sodium phosphate buffer containing 5 mM PMSF, 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂, pH 6.8) using an Amicon Ultrafree-MC 10,000 (Millipore) and was subjected to lectin affinity chromatography using ConA-Sepharose (Amersham-Pharmacia Biotech) in a batch mode. Approximately 2 ml of ConA-Sepharose equilibrated with loading buffer was mixed with enzyme in a 14 ml tube (seal cap) and tumbled end to end (8 revolution/min) for 8 h. The unbound proteins were removed by centrifugation and the resin was washed with loading buffer several times until no more protein was detected. Proteins bound to ConA-Sepharose were eluted by two washes with five column volumes of phosphate buffer containing 0.5 M methyl-α-D-mannoside. The resin was tumbled with the elution buffer overnight for the first elution and 4 h for the second elution.
The eluted fraction from ConA-Sepahrose was concentrated to 200 µl by a Ultrafree-MC (5 kDa) unit (Millipore) and applied to a FPLC size-exclusion column, Superdex 200 HR10/30 (Amersham-Pharmacia Biotech), at a flow rate of 0.4 ml/min, using 50 mM sodium phosphate buffer, pH 6.8. Fractions of 0.5 ml were collected and active fractions were pooled.

Membrane-bound α-glucosidase I was isolated from the 100,000 × g microsomal pellet by extraction for 1 h at 4°C with 200 mM sodium phosphate buffer, pH 6.8, containing 1% Brij 58, followed by centrifugation at 100,000 × g for 1h. The supernatant was applied to a Toyopearl DEAE column (2.5×10 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 0.2% Brij 58. The column was washed with the equilibration buffer plus 0.1 M NaCl, and activity was eluted with the same buffer containing 0.4 M NaCl.

### 2.3.6 Site-specific chemical modification of amino acid residues

The sensitivity of yeast α-glucosidase I to His and Cys modification was examined by diethylpyrocarbonate (DEPC) and N-ethylmaleimide (NEM), respectively. Aliquots of pure enzyme were pre-incubated with 0-10 mM of NEM and DEPC at room temperature (ca. 20°C) for 30 min. All modifications were carried out in 10 mM sodium phosphate buffer, pH 6.8. Carboxylic acid residues (i.e. Asp and/or Glu) were also subjected to specific-modification by ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDAC) by pre-incubation of enzyme with 50-150 mM EDAC at 4°C. Furthermore, the active site of enzyme was protected with 25 mM DNM prior to addition of EDAC. Activity of the treated enzyme was measured before and after dialysis against 10 mM sodium phosphate buffer, pH 6.8.

### 2.3.7 Protein sequencing
Purified soluble α-glucosidase I was electro-blotted on the Sequi-blot polyvinylidene difluoride (PVDF) membrane (0.2 μm) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 100 V for 1 h. The electro-blotting buffer was 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0. Protein was stained with 0.2% Coomassie blue in 40% methanol and destained with 50% methanol. N-terminal sequencing was conducted by the Nucleic Acid Protein Services Unit of the Biotechnology Laboratory, University of British Columbia, using standard gas phase Edman chemistry.

2.3.8 N-glycosidase F treatment

Samples for deglycosylation were dialyzed in deionised, distilled water and dried in a SpeedVac (Savant). Dried samples were dissolved in 20 mM sodium phosphate buffer, pH 7.5, containing 0.2% SDS (Fisher) and 0.5% n-octylglucoside and heated for 3 min at 95°C. After cooling, the solution of pure soluble α-glucosidase I was incubated for 20 hr at 30°C with and without N-glycosidase F (~0.5 U) (Boehringer-Mannheim).

2.3.9 Enzyme assay

α-Glucosidase I activity was assayed using the synthetic trisaccharide α-D-Glc-(1,2)-α-D-Glc-(1,3)-α-D-Glc-O-(CH$_2$)$_8$COOCH$_3$ as described previously (Neverova et al. 1994). A 4μl aliquot of enzyme solution was mixed with 1μl of 10 mM synthetic trisaccharide in microfuge tubes. Tubes were vortexed, briefly microfuged, and incubated for 5-15 min at 37°C. The reaction was quenched by adding 45μl of 1.25 M Tris-HCl, pH 7.6. The solution (50 μl) was then transferred to a microassay plate with 250 μl of developing solution, prepared according to the Sigma glucose assay kit (GAGO-20), containing glucose oxidase,
peroxidase, and O-dianisidine as the chromophore. After 30 min of incubation at 37°C in the dark, the absorbance at 450 nm was measured. One unit of enzyme activity corresponds to the amount of enzyme that produces 1 nmol of glucose per min at 37°C, pH 6.8.

Aryl α-glucosidase activity was detected using 100 mM p-nitrophenyl α-D-glucopyranoside. A 5 μl aliquot of the substrate was mixed with 15 μl of sample and incubated at 37°C for 2 to 20 h. Reactions were stopped by the addition of 300 μl of 0.2 M sodium carbonate. The absorbance of the final mixture was measured at 405 nm.

2.3.10 Other methods

Protein concentration was determined according to the method of Bradford (1976) using a Bio-Rad protein assay kit, and bovine serum albumin as the standard protein. Denaturing and reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was carried out according to the method of Laemmli (1970), using either a Mini-PROTEAN II electrophoresis cell (Bio-Rad) or a PhastSystem (Amersham-Pharmacia Biotech). Gels were stained by Coomassie blue.

2.4 RESULTS

2.4.1 Growth curve of transformed \textit{S. cerevisiae}

Transformed yeast showed no detrimental effects with respect to colony morphology but did show an increased tendency to clump during the growth period. The growth curve of the transformed yeast is shown in Figure 2.1. Preliminary screening for α-glucosidase I activity of transformed yeast from different stages of growth phase (i.e. early-log, mid-log and mid-stationary) indicated that transformed yeast at the mid-log phase, approximately 17 h after inoculation, has the highest α-glucosidase I activity per gram of
biomass. At this stage the OD$_{600}$ of the culture was approximately 4.5 and the average weight of harvested biomass was 7 g per litre of culture. The ratio of the soluble to the membrane-bound α-glucosidase I activity at this stage was 9:1. This is 9-fold higher than the previously reported ratio for the non-transformed _S. cerevisiae_ X-2180-1Bα (Kilker, Jr. et al. 1981).

2.4.2 Purification of the soluble form of α-glucosidase I

Table 2.1 provides a summary of the data obtained from different stages of purification. The specific α-glucosidase I activity after dialysis of the ammonium sulfate precipitation was determined as 19 U per mg of protein which was slightly higher than the specific activity measured from cell homogenate of 13 U per mg of protein. The soluble α-glucosidase I activity eluted from Toyopearl DEAE by 0.2 M NaCl in 10 mM sodium phosphate buffer, pH 6.8. The specific activity of enzyme was calculated as 150 U per mg of protein. This stage of purification provides an enzyme free of aryl α-glucosidase activity. Therefore, purification fold was calculated based on specific activity of the enzyme from this stage.

Optimization of the gradient elution of α-glucosidase I from the MonoQ FPLC column was effective in drastically increasing the specific activity of the enzyme to 440 U per mg of protein. α-Glucosidase I activity was eluted from the MonoQ column at approximately 0.3 M NaCl (Figure 2.2). It was observed that the soluble α-glucosidase I was tightly bound to ConA and activity was successfully eluted by 0.5 M methyl-α-D-mannoside. This is the first evidence that shows the soluble form of yeast α-glucosidase I is _N_-glycosylated. However, due to the low amount of protein recovered at this stage, concentration of protein was estimated using the theoretical extinction coefficient (URL: [link to URL]).
http://ca.expasy.org/tools/protparam.html) of the soluble enzyme, and the specific activity was estimated at 3000 U per mg of protein. SDS-PAGE analysis of the protein fraction eluted from ConA-Sepharose showed that several impurities had been co-eluted with α-glucosidase I indicating further purification was required (Figure 2.3). The last stage of the purification was carried out by FPLC size-exclusion chromatography (Superdex 200). Activity was eluted in a major peak at approximately 12.1 ml (Figure 2.4). The active fraction had an estimated specific activity of 8700 U per mg of protein and the mass of enzyme was determined to be 89 kDa. SDS-PAGE analysis of the enzyme revealed that the soluble form of enzyme has a molecular subunit mass of 98 kDa, Figure 2.5. The purity of the enzyme was estimated at ~95% based on band intensity after Coomassie blue staining (Figure 2.5).

2.4.3 Soluble α-glucosidase I is a proteolytic product of membrane-bound enzyme

N-terminal sequencing of the soluble α-glucosidase I indicated that cleavage between Ala24 and Thr25 of the membrane-bound α-glucosidase I (Cwh41p) releases the soluble activity. Interestingly, it was observed that inclusion of leupeptin and pepstatin, at 1.5 and 3.0 μg/ml, respectively, during the enzyme extraction did reduce the amount of the soluble enzyme obtained from a preparation approximately 2-fold, but did not completely prevent its release. Furthermore, PMSF at 85-100 μg/ml did not affect the release of the soluble α-glucosidase I activity.

2.4.4 Soluble α-glucosidase I is N-glycosylated

The soluble α-glucosidase I activity is bound to ConA and eluted with a high concentration of methyl-α-D-mannoside (0.5 M) indicating that the soluble α-glucosidase I is
an *N*-glycoprotein, which is in agreement with previous studies that reported the membrane form of the enzyme is *N*-glycosylated (Bause et al. 1986; Jiang et al. 1996). Furthermore, it was found that treatment of the soluble α-glucosidase I with N-glycosidase F did reduce the molecular weight of the subunit by approximately 5 kDa as determined by SDS-PAGE (Figure 2.6).

### 2.4.5 Chemical modification of amino acid residues

The yeast α-glucosidase I was not sensitive to NEM, as no inhibition was observed at 5 mM; however the enzyme activity was inhibited by DEPC, with 60% inhibition at 10 mM DEPC (Table 2.2). In addition, EDAC was found to inhibit α-glucosidase I activity in a time- and concentration-dependent manner (Figure 2.7 A). Maximum inhibition (ca. 90%) was observed when α-glucosidase I was treated with 150 mM EDAC for 60 min at 4°C. Approximately 75% of the activity was recovered when enzyme was pre-incubated with 25 mM DNM (Genzyme) prior to addition of EDAC (Figure 2.7 B). Chemical reagents at the concentration employed in this study did not interfere with the coupling reaction used for measuring α-glucosidase I activity.

### 2.5 DISCUSSION

Purification of the soluble α-glucosidase I to approximately 95% homogeneity, based on SDS-PAGE analysis, was achieved by a combination of ammonium sulfate precipitation (20-60%), anion-exchange, ConA affinity, and size-exclusion chromatographies. The molecular mass of the soluble α-glucosidase I was 98 kDa in a reduced form by SDS-PAGE and approximately 89 kDa by gel-filtration. The discrepancy between molecular weight of the soluble α-glucosidase I by SDS-PAGE and gel-filtration is
likely due to an interaction of the enzyme with dextran of the Superdex 200 column which resulted in delaying in the elution. Indeed, similar differences in molecular mass measurement carried out by SDS-PAGE and Superdex 200 gel-filtration have been reported for the other glycosidases such as β-glucosidase (Li et al. 2005), plant endo-β-mannosidase (Ishimizu et al. 2004), recombinant β-(1,3)-xylanase (Araki et al. 2000) and hen α-(1,2)-mannosidase (Hamagashira et al. 1996). However, inclusion of 0.5 M α-D-glucose in the elution buffer only slightly decreased the retention volume of the soluble α-glucosidase I from Superdex 200 column. The molecular subunit mass of the soluble α-glucosidase I under these conditions was determined to be 93 kDa. This suggests that the soluble yeast α-glucosidase I, unlike its mammalian counterpart, is a monomeric protein.

Based on protein sequence, the membrane-bound α-glucosidase I has a predicted molecular mass of 96,512 Da, where as the molecular mass of the region Thr25-Phe833 is calculated as 93,800 Da. The molecular mass of 98 kDa for the soluble α-glucosidase I reported here is slightly higher than that of the membrane-bound enzyme of 95 kDa reported by Bause and co-workers (1986).

Based on N-terminal sequencing analysis, a significant increase in the soluble α-glucosidase I activity in yeast overexpressing CWH41, the molecular mass of the purified soluble α-glucosidase I, and partial inhibition of the soluble α-glucosidase I formation by leupeptin and pepstatin, it was demonstrated that the soluble α-glucosidase I is part of Cwh41p and is likely released when the cells are broken at the beginning of the isolation protocol. The cleavage site, Ala24-Thr25, is located at the end of the predicted transmembrane domain (Thr11- Ile28) of Cwh41p, close to the lumen of the ER. Interestingly this cleavage site matches with the theoretical signal-peptide cleavage site for Cwh41p (SignalP 3.0 server, URL: http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al.
A similar proteolytic cleavage of membrane-bound glycosyltransferases with release of a soluble form has been reported to occur in the Golgi (Jaskiewicz et al. 1996; Lammers & Jamieson 1988).

Yeast α-glucosidase I contains five potential N-glycosylation sites (Figure 1.4 A). Deglycosylation of enzyme with N-glycosidase F was concomitant with reducing the molecular subunit mass by about 5 kDa suggesting two out of five predicted glycosylation sites are likely N-glycosylated.

Incubation of pure enzyme with 0.5-25 mM kojibiose over an extended period of time did not release any glucose and therefore it is not a substrate for the soluble α-glucosidase I. Indeed, pre-treating the enzyme with 5 mM kojibiose inhibited the release of glucose from the synthetic substrate, showing that it is an inhibitor for the soluble α-glucosidase I. This result is in agreement with the previous report that showed kojibiose inhibited membrane-bound yeast α-glucosidase I with the Ki of 55 μM. However, the Ki was determined at an unreported level of [14C] Glc₃Man₉GlcNAc₂ (Bause et al. 1986). Therefore, three glucose residues, as found in the synthetic trisaccharide substrate used in this work, are the minimal structure required for catalysis; however, the influence of the hydrophobic aglycone on binding cannot be discounted since a hydrophobic pocket has been suggested to be present at the catalytic site (Bause et al. 1986).

α-Glucosidase I from mammalian and plant sources exhibited distinct differences between the sensitivity to modification of His by DEPC and Cys residues by NEM. Enzyme from mung beans showed sensitivity to DEPC treatment, resulting in 95% inhibition while NEM treatment resulted in only 20% inhibition of the activity (Zeng & Elbein 1998). In contrast, α-glucosidase I from pig liver (Zeng & Elbein 1998) and rat liver (Romaniouk & Vijay 1997) showed the opposite trend. Yeast α-glucosidase I was not sensitive to NEM as
no inhibition was observed at 10 mM; however the activity was inhibited by DEPC, with 60% inhibition observed at 10 mM. Therefore, the yeast enzyme appears to be more similar to the mung bean enzyme. Romaniouk and Vijay (1997) have suggested that Glu594-Trp602 (E594RHLDLRCW602) of the human hippocampus enzyme, which corresponds to Glu613-Trp621 (E613LNVDALAW621) of the yeast enzyme, serves as the binding motif for the α-glucosidase I. In the mammalian sequence, a Cys residue is present at position 601, and it may responsible for the sensitivity of the mammalian forms of the enzyme to NEM. There is no Cys residue in the corresponding sequence of the yeast enzyme, which may explain the resistance of the yeast enzyme to inhibition by NEM. Modification of the enzyme with the site-specific reagent for carboxylic acid residues, EDAC, indicated that α-glucosidase I is inhibited in a time- and concentration-dependent manner. Greater than 90% inhibition of activity after 60 min incubation with 150 mM EDAC was observed, which could be prevented when the enzyme was protected with 25 mM DNM. It has been established that the catalytic activity of glycosidases is based on carboxylic acid residues which are involved in general acid-base catalysis of the substrate (Rye & Withers 2000). Therefore, the data suggests that there is at least one catalytically active carboxylic acid residue in yeast α-glucosidase I that is susceptible to chemical modification.

2.6 FUTURE STUDIES

Purification of the soluble α-glucosidase I to ~95% of homogeneity was achieved by a combination of anion exchange, lectin affinity, and size-exclusion chromatographies. However, enzyme was purified with a low yield of ~1%, likely due to large number of purification steps. For further characterization studies, it is essential to acquire a larger quantity of the enzyme with higher purity. To achieve this, it is necessary to obtain a larger
biomass of transformed yeast, to optimize current methods, and to improve the purification method replacing some of the stages with more selective methods such as DNM-based affinity chromatography.

Preliminary results from this study revealed that the yeast enzyme is sensitive to His modification; however it is yet to be determined if a His residue is involved in catalytic activity or has a structural implication for the enzyme. Further study is also required to examine the function of other important residues (i.e. Arg, Trp and Tyr), as these have been reported to be critical for the mammalian enzyme (Romaniouk & Vijay 1997).
Figure 2.1. Growth curve of *S. cerevisiae* overexpressing CWH41. Growth curve of transformed yeast in the incubator shaker (300 rpm, 28°C) was determined by measuring turbidity of the media at A600 nm at 1 h time intervals. Each point represents the average measurement of two cultures.
Table 2.1. Purification of the soluble α-glucosidase I isolated from transformed *S. cerevisiae* overexpressing *CWH41*.  

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Homogenate</td>
<td>310±46</td>
<td>23±3.6</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>20-60% (NH₄)₂SO₄</td>
<td>380±42</td>
<td>20±0.3</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>Toyopearl DEAE</td>
<td>310±92</td>
<td>2.1±0.2</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>FPLC MonoQ HR5/5</td>
<td>110±46</td>
<td>0.25±0.1</td>
<td>440</td>
<td>3</td>
</tr>
<tr>
<td>ConA-Sepharose</td>
<td>34±9</td>
<td>0.011±0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3100</td>
<td>20</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>2.7±0.3</td>
<td>0.31×10⁻³±0.1×10⁻³&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8700</td>
<td>58</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are the average ± range of two preparations and reported per gram wet weight of yeast biomass

<sup>b</sup> 1 U of activity = releasing 1 nmol of glucose per min.

<sup>c</sup> Protein was estimated from the theoretical extinction coefficient of 150,000 M⁻¹cm⁻¹ (at 280 nm) and a molecular weight of 93.8 kDa.
Figure 2.2. Elution of the soluble α-glucosidase I activity from the MonoQ FPLC. The peak containing α-glucosidase I activity is indicated by the arrow. The solid line is the absorbance at 280 nm, and the dashed line is the percentage of 1 M NaCl in 10 mM sodium phosphate buffer, pH 6.8.
Figure 2.3. SDS-PAGE (8-25%, PhastGel) of proteins eluted from ConA-Sepharose. Lane 1, α-glucosidase I activity eluted by 0.5 M methyl-α-D-mannoside; lane 2, molecular weight standards.
Figure 2.4. Elution of α-glucosidase I from the Superdex 200 FPLC. α-Glucosidase I is eluted at approximately 12.1 ml. The solid line represents the elution profile of soluble α-glucosidase I and the dotted line is the elution profile of protein standards; the value above each peak shows the molecular weight of the corresponding standard protein in kDa.
Figure 2.5. SDS-PAGE (8-25%, PhastGel) of the soluble $\alpha$-glucosidase I after gel-filtration chromatography. Lane 1, $\alpha$-glucosidase I; lane 2, molecular weight standards.
Figure 2.6. SDS-PAGE (12.5%) of the soluble α-glucosidase I treated with N-glycosidase F. α-Glucosidase I after overnight incubation at 30°C with (lane 2) and without (lane 1) N-glycosidase F. Lane 3, molecular weight standards (Bio-Rad).
Table 2.2. Inhibition of the soluble α-glucosidase I by NEM and DEPC.

<table>
<thead>
<tr>
<th></th>
<th>Remaining α-glucosidase I activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>NEM</td>
<td>115</td>
</tr>
<tr>
<td>DEPC</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup>Results are the average of two experiments. Enzyme without (control) and with reagents were incubated for 30 min at room temperature (ca. 20°C).
Figure 2.7. $\alpha$-Glucosidase I activity modification with EDAC. (A) Enzyme in 10 mM sodium phosphate buffer pH 6.8 was treated with 50 (▲), 100 (■), and 150 (◆) mM EDAC and incubated for 20, 40 and 60 min at 4°C. Results are presented as the percentage of remaining activity compared to the control. (B) Enzyme was treated with (a) 150 mM EDAC, (b) 25 mM DNM, and (b) 25 mM DNM, then 150 mM EDAC for 1 hr at 4°C. Remaining activity was measured before (□) and after (■) overnight dialysis against 10 mM sodium phosphate buffer, pH 6.8, at 4°C. Results are presented as the remaining activity compared to the appropriate control for two independent experiments. Error bars represent the range of the two measurements.
CHAPTER III

PART 2. PURIFICATION OF THE SOLUBLE $\alpha$-GLUCOSIDASE I TO HOMOGENEITY
Chapter III

Preface

A version of Chapter III has been published in Protein Expression and Purification 2004.

3.1 ABSTRACT

Processing $\alpha$-glucosidase I, which is encoded by *CWH41*, regulates the first steps in asparagine-linked glycoprotein biosynthesis by cleaving the terminal glucose from Glc$_3$Man$_9$GlcNAc$_2$, the common oligosaccharide precursor. A method is described for the purification of the soluble form of $\alpha$-glucosidase I to homogeneity from *Saccharomyces cerevisiae* overexpressing *CWH41*. A homogeneous enzyme preparation was obtained in higher yield than the previous method. Cultivation of transformed *Saccharomyces cerevisiae* in a fermenter increased the biomass 1.7 times per litre and enzyme production 2 times per litre compared to cultivation in shake flasks. Ammonium sulfate precipitation followed by three chromatography steps, including chromatography on an $N$-(5'-carboxypentyl)-1-deoxynojirimycin column, resulted in highly purified enzyme with no detectable contamination by aryl $\alpha$- and $\beta$- glucosidase. The purification procedure reproducibly yielded 40 $\mu$g of pure enzyme per g wet biomass. Enzyme that was purified using an alternative procedure (described in Chapter II) contained minor impurities, and was hydrolyzed by an endogenous proteolytic activity to peptides that retained full catalytic activity. Controlled trypsin hydrolysis of the highly purified enzyme released polypeptide(s) containing the $\alpha$-glucosidase I catalytic domain, with no loss of catalytic activity. This suggests that the catalytic domain of yeast $\alpha$-glucosidase I is resistant to trypsin hydrolysis and remains fully functional after cleavage.
3.2 INTRODUCTION

In yeast, like other eukaryotes, assembly and early processing of carbohydrates to produce an N-linked glycoprotein takes place in the lumen of the ER (Herscovics 1999b). Removal of glucose residues by processing α-glucosidase I and II is reported to be crucial for protein quality control (QC) in the endoplasmic reticulum (ER) (Helenius & Aebi 2004). In some eukaryotic cells, improperly folded glycoproteins can be retained in the ER after being re-glucosylated to Glc1Man9GlcNAc2 by glucosyltransferase and interaction with calnexin or calreticulin, to allow them to potentially attain the proper conformation (Parodi 2000a; Spiro 2000). While the process of glycoprotein QC has not been clearly determined in S. cerevisiae, monoglucosylated proteins may be involved (Jakob et al. 1998b). Recently it has been reported that the S. cerevisiae mutant (derl-) lacking α-glucosidase I activity was unable to degrade misfolded glycoproteins, emphasizing an important role of α-glucosidase I in the QC of glycoproteins in yeast (Hitt & Wolf 2004). Moreover, S. cerevisiae mutant strains with impaired α-glucosidase I activity have a reduced cell wall β-(1,6)-glucan content (Jiang et al. 1996; Simons et al. 1998). Further studies suggest that α-glucosidase I has an indirect effect on β-(1,6)-glucan biosynthesis likely by regulating other N-glycoproteins that may be directly involved in biosynthesis of yeast cell wall (Abeijon & Chen 1998). In higher eukaryotes several studies have demonstrated that inhibition of α-glucosidase I activity with DNM or castanospermine alters glycoprotein accumulation and degradation, and therefore cell surface expression of glycoproteins (Arakaki et al. 1987; Edwards et al. 1989; Elbein 1991).

In S. cerevisiae, CWH41 encodes Cwh41p (processing α-glucosidase I), which is a type II integral membrane glycoprotein located in the ER (Jiang et al. 1996; Romero et al. 1997). From the predicted sequence, membrane-bound α-glucosidase I (Cwh41p) contains
833 amino acid residues with the theoretical mass of 96.5 kDa. It is purported to have a short N-terminal cytosolic tail (Met1-Lys10), a large C-terminal catalytic domain (Ser29-Phe833) extending toward the lumen of the ER, with a short hydrophobic transmembrane region (Thr11-Ile28) linking the two domains (URL: http://ca.expasy.org/cgi-bin/niceprot.pl?P53008).

In the previous chapter a purification method that achieved approximately 95% homogeneity of the soluble form of the yeast enzyme, using anion-exchange, ConA, and gel-filtration chromatographies, was described. However, this method purifies the soluble α-glucosidase I with a low yield of approximately 1% of the starting activity. It was demonstrated that the soluble form of enzyme activity was generated by proteolytic cleavage between residues Ala 24 and Thr 25, the theoretical signal-peptide cleavage site at the predicted transmembrane domain of Cwh41p. This clipping releases the luminal C-terminal catalytic domain of the enzyme (Thr25-Phe833) from the membrane-bound enzyme. It was also shown that the soluble α-glucosidase I is an N-linked glycoprotein.

Several studies have reported that the membrane-bound α-glucosidase I can be purified from different eukaryotic cells using DNM-based resins (Bause et al. 1986; Bause et al. 1989; Hettkamp et al. 1984; Shailubhai et al. 1987; Zeng & Elbein 1998). Therefore this study was primarily focused on increasing the purification yield and homogeneity of enzyme. This was achieved by increasing biomass and activity of the transformed yeast by growing recombinant S. cerevisiae in a fermenter and using CP-DNM affinity chromatography. The improved four-step purification method as described here purifies the soluble α-glucosidase I more rapidly, to a higher degree of homogeneity, and to a higher yield than the previous method described in Chapter II. Furthermore, formation of catalytically active polypeptides by endogenous proteolytic activity and by controlled proteolysis with trypsin is described.
3.3 MATERIALS AND METHODS

3.3.1 Materials

Chemicals used for the purification of α-glucosidase I were reagent grade, except where noted. Sequencing grade modified trypsin was obtained from Promega, and N-glycosidase F was obtained from Boehringer-Mannheim. DNM-HCl salt was purchased from Genzyme and the glucose assay kit (GAGO-20) was a product of Sigma. Unless stated, all chemicals were obtained from Sigma.

3.3.2 Synthesis of CP-DNM resin

The CP-DNM was synthesized at the Department of Chemistry, University of Alberta as described previously (Bernotas & Ganem 1990). The synthesized CP-DNM was covalently linked to AH-Sepharose 4B through the water-soluble carbodiimide-mediated amidation (Sehgal & Vijay 1994).

3.3.3 Yeast strain, media, and growth

*S. cerevisiae* overexpressing *CWH41* (section 2.3.3) was used in this study. Yeast transformants were grown in medium containing 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% glucose, and 50 mg/l histidine. Two ml of an overnight culture was inoculated into each of four 1000 ml flasks containing 250 ml fresh media. After 17 h incubation at 28°C with shaking at 300-350 rpm, a final OD$_{600}$ of approximately 4.5 was obtained and used for inoculation of 4 litre of media (20% V/V) in a glass stirred-tank fermenter (VirTis Co.). The cells were harvested after 18 h cultivation at 28°C, 400 rpm, and an aeration rate of 1.9 l/min.
3.3.4 Enzyme isolation and purification

All steps for enzyme isolation were carried out at 4°C. Throughout enzyme isolation and purification 5 mM phenylmethylsulfonylfluoride (PMSF) was added to the active fractions that were pooled after each step. Yeast cells were separated from culture by centrifugation at 8,000 × g for 15 min, and the cell pellet was washed with 20 mM sodium phosphate buffer, pH 6.8. Yield of biomass harvested from 5 litre of media was approximately 51 g. The packed cells were resuspended in three volumes of the 20 mM sodium phosphate buffer, pH 6.8 with 5 mM PMSF and four volumes of chilled, sterilized, acid-washed glass beads. Yeast cells were broken by homogenizing for six minutes at high speed in a Bead Beater (Biospec Products Inc.), with cooling on ice for 1 to 2 min after every minute of homogenization. The supernatant was decanted from the glass beads and the beads were washed with three volumes of the same phosphate buffer. The supernatants were pooled and centrifuged for 25 min at 15,000 × g. Microsomal pellets were isolated as described in section (2.3.4); the supernatant was used for the isolation of the soluble α-glucosidase I and microsomes for the membrane-bound α-glucosidase I. The soluble α-glucosidase I activity was precipitated and isolated using 20-60% ammonium sulfate saturation as described in the previous study. The pellet was dissolved in 90 ml of 20 mM sodium phosphate buffer, pH 6.8, and dialyzed against 4 litre of the same buffer for approximately 24 h, with one buffer change at 12 h.

The dialyzed sample was applied to a Toyopearl DEAE column (2.5 × 20 cm) equilibrated with 20 mM phosphate buffer (pH 6.8) with a flow rate of 2 ml/min. The unbound proteins were removed from the column by washing with five column volumes of the same buffer. Proteins were eluted using a step-wise gradient with 0.1 M and 0.2 M NaCl in 20 mM sodium phosphate buffer, pH 6.8, with a flow rate of 2-2.5 ml/min. Fractions of 15
ml were collected during the 0.2 M NaCl elution. Active fractions were pooled and dialyzed for 8 h in 4 litre 20 mM sodium phosphate buffer, pH 6.8, and then concentrated with an Amicon 8050 Ultrafiltration with a Ultracel YM10 membrane (Millipore). The concentrated sample was applied to an FPLC MonoQ HR5/5 column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium phosphate buffer, pH 6.8 (loading buffer). An optimized continuous gradient elution ranging from 0-1.0 M NaCl in loading buffer with a flow rate of 0.4 ml/min as described in the previous chapter was used to elute proteins. Fractions of 1.0 ml were collected and the fractions with activity were pooled.

Enzyme was applied to the CP-DNM column (1.5 x 20 cm) equilibrated with 0.5 M NaCl in 100 mM sodium phosphate buffer, pH 6.8 (loading buffer) at a flow rate of 100-150 μl/min. Two distinct peaks of protein were eluted with the same loading buffer and collected separately. To ensure complete removal of all unbound proteins, the resin was washed with a further 15 volumes of loading buffer. Elution of the tightly bound α-glucosidase I activity was conducted with 200 mM trehalose in loading buffer as described by Zeng and Elbein (1998), using 5 volumes of 200 mM trehalose in loading buffer. This trehalose-eluted fraction was dialyzed overnight in 20 mM sodium phosphate buffer, pH 6.8, with three changes of buffer.

Membrane-bound α-glucosidase I was isolated and partially purified from the microsomes of transformed yeast according to the method described in the previous chapter.

3.3.5 Lectin affinity chromatography

Lectin affinity chromatography was performed by applying the MonoQ eluted, dialyzed sample in loading buffer (20 mM phosphate buffer, pH 6.8 plus 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂) onto a column (1 x 10 cm) of ConA-Sepharose (Amersham Pharmacia
Biotech) at a flow rate of 0.35 ml/min. Unbound proteins were removed by washing with ten resin volumes of loading buffer. Bound proteins were eluted stepwise with five resin volumes each of 20 mM phosphate buffer, pH 6.8, containing 0.5 M NaCl and 0.1 M methyl-\(\alpha\)-D-mannoside, followed by the same phosphate buffer containing 0.5 M NaCl and 0.3 M methyl-\(\alpha\)-D-mannoside.

### 3.3.6 Enzyme assay

\(\alpha\)-Glucosidase I activity was measured using synthetic trisaccharide. The assay is described in detail in section (2.3.9). Aryl \(\alpha\)- and \(\beta\)-glucosidase activities were determined using 100 mM \(p\)-nitrophenyl \(\alpha\)- and \(\beta\)-D-glucopyranoside, respectively, as described in section 2.3.9. The absorbance of the final mixture was measured at 405 nm. One enzyme unit is defined as the amount of enzyme that cleaves 1 \(\mu\)mol substrate per minute (\(\varepsilon\) for \(p\)-nitrophenol, \(1.77 \times 10^4\) M\(^{-1}\)cm\(^{-1}\)).

### 3.3.7 Other methods

Deglycosylation of purified protein was conducted using \(N\)-glycosidase F as described in section 2.3.8. Protein concentration was determined according to the method of Bradford (1976) using a Bio-Rad protein assay kit, and bovine serum albumin as the standard protein. SDS-PAGE analysis was carried using the method of Laemmli (1970) as described in section 2.3.10. All of the gels were stained using Coomassie blue. Bio-Rad Mini Trans-Blot system was used for protein blotting onto PVDF membranes, as described in section 2.3.7. The protein sequence of blotted samples was determined by the Nucleic Acid Protein Services Unit, Biotechnology Laboratory, University of British Columbia, using standard gas phase Edman chemistry.
3.4 RESULTS

3.4.1 Growth and activity of transformed yeast in fermenter

Batch mode growth of recombinant *S. cerevisiae* using a fermenter significantly improved the production of biomass to 10±0.8 g (wet basis) per litre of culture, compared to 6.2±0.9 g/l of culture previously obtained from shaking flask incubation. α-Glucosidase I activity per litre was increased 2 times using the fermenter, as calculated from the dialyzed ammonium sulfate pellet. The ratio of the soluble α-glucosidase I to the membrane-bound activity was determined to be 9.6:1. This ratio is in close agreement with the previous result of 9:1 from the same transformed yeast cells which were grown in flasks.

3.4.2 Purification of the soluble α-glucosidase I

Purification of the soluble α-glucosidase I to homogeneity was achieved by a combination of ammonium sulfate precipitation, anion exchange chromatography, and CP-DNM chromatography (Table 3.1). The yield of purified α-glucosidase I was 26%, or approximately 40 μg of the homogeneous soluble enzyme per g of recombinant *S. cerevisiae* biomass. Figure 3.1 shows the enzyme purity at each step.

It was previously demonstrated that the soluble α-glucosidase I activity was eluted from Toyopearl DEAE by 0.2 M NaCl in phosphate buffer, pH 6.8. However, it was noted that elution of the enzyme activity from Toyopearl DEAE chromatography was limited to the relatively restricted volume of this fraction. Using the fractions from 70 ml to 150 ml (Figure 3.2) increased the specific activity 3-fold compared to using the whole 0.2 M elution. This stage of purification provides an enzyme preparation free of aryl α-glucosidase activity.

The concentrated enzyme from the Toyopearl DEAE was applied to the MonoQ HR 5/5 anion exchange FPLC column. A continuous gradient elution resulted in a major eluted
peak at 0.3 M NaCl in phosphate buffer, pH 6.8, which corresponded to enzyme activity (Figure 3.3). This fraction had a relatively high degree of purity (Figure 3.1, lane 5) with a specific activity of 1650 U/mg protein which was 4 times that of the previous study.

The enzyme from the MonoQ column was then applied to the CP-DNM resin. Two distinct peaks were separated from the resin by the loading buffer (Figure 3.4 A). The first peak (Peak I) included many proteins (Figure 3.4B, lane 1) and did not have any α-glucosidase I activity. The second peak (Peak II) was a single protein band (Figure 3.4 B, lane 3) with an approximate molecular mass of 98 kDa by SDS-PAGE that contained 120 units α-glucosidase I activity/g biomass (36% of the loaded activity) with a specific activity of 3000 U/mg protein. This pure fraction of the soluble α-glucosidase I is an N-glycoprotein, as shown by N-glycosidase F treatment (Figure 3.5), and as previously noted. The specific activity for Peak II α-glucosidase I was 2.7 times lower than the specific activity of enzyme reported in the previous method that contained minor contaminants. This discrepancy is likely attributed to use of the protein’s theoretical extinction coefficient to estimate the specific activity in the later stages of purification used in the earlier report, rather than the actual measurement of protein concentration that was used in this study.

The bound proteins that were eluted by 200 mM trehalose from the CP-DNM column had a total activity of 31 U/g of biomass, which was 9.4% of the loaded activity, and specific activity of 2100 U/mg of protein. The SDS-PAGE of eluted proteins (Figure 3.4 B, lane 5) showed three major proteins with the approximate masses of 98, 58, and 38 kDa respectively. The trehalose-eluted fractions had ~22 mU aryl β-glucosidase activity/mg protein, while no aryl β-glucosidase activity was detected in the pure α-glucosidase I fraction (Peak II). Aryl β-glucosidase activity (81 mU/mg protein) was also detected in Peak I from the CP-DNM column.
To compare the efficiency of the newly established purification method with the previous method (Chapter II), another enzyme preparation, after ammonium sulfate, Toyopearl DEAE, and FPLC Mono Q chromatography, was applied to ConA-Sepharose and the majority of α-glucosidase I activity was eluted by 0.3 M methyl-α-D-mannoside. Pooled active fractions were concentrated and applied to a Superdex 200 gel-filtration FPLC column, as described previously (section 2.3.5). The eluted α-glucosidase I from gel-filtration had specific activity of 2200 U/mg protein but several minor impurities co-eluted with it. A small amount of aryl β-glucosidase activity was present in this fraction. To determine the source of this activity, a sample of α-glucosidase I eluted from gel-filtration was applied to the CP-DNM resin and bound proteins were eluted by 50 mM DNM in a batch mode. After dialysis against 20 mM sodium phosphate buffer, pH 6.8, the eluted sample was found to contain the aryl β-glucosidase activity. SDS-PAGE of the eluted proteins showed that the major protein, other than α-glucosidase I, was a 59 kDa protein (Figure 3.6). N-terminal sequencing of this protein identified it as exo-1,3-β-glucanase I/II (EC 3.2.1.58), a glycoprotein involved in cell wall β-1,3- and β-1,6-glucan metabolism (Jiang et al. 1995). Therefore, the combination of ConA-Sepharose and gel-filtration chromatography could not separate all aryl β-glucosidase activities from the α-glucosidase I preparation.

3.4.3 Endogenous and trypsin hydrolysis of the soluble α-glucosidase I

Storage of the partially purified enzyme, obtained using the ConA and gel-filtration chromatography, for one month at 4°C resulted in complete loss of the 98 kDa α-glucosidase I band, the generation of two intense bands at 58 and 38 kDa, and the formation of two minor bands at 32 and 29 kDa (Figure 3.7). Despite the loss of the 98 kDa band, the α-glucosidase I activity was 1.1 times that of the original activity, suggesting that either one or several of the
peptides contained an active catalytic fragment that was resistant to further degradation. In contrast, the purified enzyme (Peak II) was stable for at least 2 months at 4°C, indicating that it did not contain the endogenous proteolytic activity.

Formation of active polypeptide(s) during storage of α-glucosidase I raised the question as to whether other proteases, such as trypsin, could produce active fragments from the homogeneous soluble α-glucosidase I. Therefore, freshly purified α-glucosidase I was hydrolyzed with trypsin at 4°C for 2 days. Hydrolysis of α-glucosidase I with trypsin produced polypeptides with molecular weights of 59, 37, 32, and 29 kDa (Figure 3.8), which had an activity that was 1.1 times that of the original enzyme solution.

3.5 DISCUSSION

The yeast fermentation technique and enzyme isolation described here resulted in an improved yield and purification of the soluble form of α-glucosidase I compared to the previous study (Table 3.2).

Using the CP-DNM resin in the purification process was crucial for obtaining the homogeneous soluble α-glucosidase I. The soluble form of α-glucosidase I had a weak affinity for the CP-DNM resin and could be eluted as a pure protein from the column with buffer B (Peak II) while Bause and co-workers (1986) reported the successful binding of membrane-bound α-glucosidase I from *S. cerevisiae* to the same resin. However, they also reported that the specific elution of the membrane-bound forms of the yeast enzyme from the CP-DNM resin contained impurities, which required further purification (Bause et al. 1986). The weak binding of the soluble form of the enzyme to the CP-DNM resin, in contrast to the membrane enzyme, could be the result of the altered hydrophobicity of the soluble enzyme due to cleavage of the transmembrane segment, or due to the differences in the binding buffer
used here, which lacked detergent, and had a different salt concentration and pH. It is also possible that the column binding technique that was used here, rather than the batch mode reported, may have affected binding. During this study, a reduction in the enzymatic activity (up to 70%) was observed during batch mode binding of the enzyme to resin, possibly due to increased shear stress caused by stirring or tumbling, as compared to using a column mode.

α-Glucosidase I with a lower degree of purity was endogenously hydrolyzed to small active fragments that retained the same level of activity as the intact enzyme. Using trypsin, active polypeptides were also formed without loss of activity. Other studies have reported the formation of catalytically active polypeptides from mammalian sources of processing α-glucosidase I treated with trypsin (Bause et al. 1989; Shailubhai et al. 1991). The formation of three major polypeptides (either 69, 55, and 39 kDa, or 69, 45, and 29 kDa) resistant to trypsin hydrolysis from several sources of mammalian membrane-bound α-glucosidase I has been reported (Pukazhenthi et al. 1993b). The results of trypsin proteolysis in this study, and as reported by others for mammalian α-glucosidase I (Bause et al. 1989; Shailubhai et al. 1991) suggests that the catalytic domain of these orthologs is likely resistant to proteolysis.

3.6 FUTURE STUDIES

Results of this study showed that the soluble α-glucosidase I can be purified with relatively high yield (26%) and homogeneity which provides sufficient quantity of pure enzyme to conduct further characterization of the enzyme. Moreover, formation of active polypeptides that can be liberated from the soluble α-glucosidase I draws attention to the possibility of isolation and characterization of these active fragments containing putative catalytic domain.
Table 3.1. Purification of the soluble α-glucosidase I from recombinant *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Activity (U)(^b)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 – 60% (NH(_4))(_2)SO(_4)</td>
<td>460 ±21</td>
<td>12±2.9</td>
<td>38±4</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Toyopearl DEAE</td>
<td>450±90</td>
<td>0.9±0.2</td>
<td>500±10</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>FPLC Mono Q HR5/5</td>
<td>330±44</td>
<td>0.2±0.05</td>
<td>1650±210</td>
<td>72</td>
<td>3.3</td>
</tr>
<tr>
<td>CP-DNM</td>
<td>120±8</td>
<td>0.04±0.001</td>
<td>3000±130</td>
<td>26</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) Results are the average ± range of two preparations and reported per gram wet weight of yeast cells (multiple values by 51 g biomass to obtain total protein and activity in a typical 5 litre batch of fermentation).

\(^b\) 1 U of activity = 1 nmol of released glucose per min.
Figure 3.1. SDS-PAGE (12.5%) of α-glucosidase I fractions at different stages of purification. Lane 1 and 7, molecular weight standards; lane 2, cell homogenate; lane 3, 20-60% ammonium sulfate precipitate; lane 4, enzyme after Toyopearl DEAE; lane 5, enzyme fraction after the MonoQ HR5/5; lane 6, pure enzyme from CP-DNM chromatography (Peak II α-glucosidase I). Lanes 2-5 were loaded with 15 μg of protein and lane 6 was loaded with 4 μg of protein.
Figure 3.2. The 0.2 M NaCl elution profile of α-glucosidase I activity from Toyopearl DEAE. The solid line is protein concentration (mg/ml), and the dashed line is the α-glucosidase I activity (U/ml). Proteins applied in 20 mM phosphate buffer, pH 6.8 to column and were eluted by step gradient of 0, 0.1, and 0.2 M NaCl in 20 mM phosphate buffer, pH 6.8. Only the 0.2 NaCl profile is shown.
Figure 3.3. Elution of α-glucosidase I from the MonoQ HR5/5 column. The solid line is the absorbance at 280 nm, and the dotted line is the percentage of loading buffer (20 mM phosphate buffer, pH 6.8) containing 1M NaCl. The major peak contained α-glucosidase I activity.
Figure 3.4. Chromatography with the CP-DNM resin. (A) Chromatogram of unbound protein (Peak I) and soluble \( \alpha \)-glucosidase I (Peak II) eluted from the CP-DNM column with loading buffer (0.5 M NaCl in 100 mM sodium phosphate buffer, pH 6.8). (B) SDS-PAGE of the CP-DNM resin fractions. Lane 1- Peak I (20 \( \mu \)g); lane 3 - Peak II \( \alpha \)-glucosidase I (4 \( \mu \)g); lane 5 - proteins eluted with 200 mM trehalose (12 \( \mu \)g). Lanes 2 (Bio-Rad), 4, and 6 (Sigma) are molecular weight standards.
Figure 3.5. SDS-PAGE (12.5%) of deglycosylated soluble α-glucosidase I. Peak II α-glucosidase I with (Lane 1) and without (Lane 2) N-glycosidase F treatment. Lane 3, 2 µg each from N-glycosidase F treated and untreated sample.
Figure 3.6. SDS-PAGE (10-15%, PhastGel) of proteins purified according to the method described in Chapter II, followed by DNM-elution from CP-DNM resin. The arrow shows the band identified as exo-1,3-β-glucanase I/II. Lane 2, molecular weight standards (Sigma).
Figure 3.7. SDS-PAGE (15%) of endogenously degraded $\alpha$-glucosidase I, isolated according to the method described in Chapter II. Lane 1, polypeptides with $\alpha$-glucosidase I activity. Lane 2, molecular weight standards (Sigma).
Figure 3.8. SDS-PAGE (8-25% PhastGel) of tryptic polypeptides of Peak II α-glucosidase I. 10 μg of α-glucosidase I was mixed with 2 μg of sequencing grade trypsin and kept at 4°C for 2 days. Lane 1, Polypeptides (3μg, arrow shows trypsin). Lane 2, molecular weight standards.
Table 3.2. Comparison of purification methods for the soluble yeast α-glucosidase I.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Data from Chapter II</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Yield</td>
</tr>
<tr>
<td></td>
<td>(U/mg)</td>
<td>(%)</td>
</tr>
<tr>
<td>20 – 60% (NH₄)₂SO₄</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>Toyopearl DEAE</td>
<td>150</td>
<td>81</td>
</tr>
<tr>
<td>FPLC MonoQ HR5/5</td>
<td>440</td>
<td>29</td>
</tr>
<tr>
<td>CP-DNM</td>
<td>NA c</td>
<td>NA</td>
</tr>
<tr>
<td>ConA-Sepharose</td>
<td>3100 b</td>
<td>9</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>8700 b</td>
<td>1</td>
</tr>
</tbody>
</table>

\(a\) Specific activity= units of enzyme per mg of protein.  
\(b\) Protein concentration was estimated based on theoretical extinction coefficient.  
\(c\) NA-chromatographic step was not applied in this purification method.
CHAPTER IV

PART 3. CATALYTIC DOMAIN AND BINDING RESIDUES OF THE
SOLUBLE α-GLUCOSIDASE I
4 CHAPTER IV

Preface

A version of Chapter IV has been accepted for publication in Glycobiology 2005.

4.1 ABSTRACT

a-Glucosidase I initiates the trimming of newly assembled N-linked glycoproteins in the lumen of the endoplasmic reticulum. Site-specific chemical modification of the soluble a-glucosidase I from yeast using diethylpyrocarbonate and tetranitromethane revealed that histidine and tyrosine are involved in the catalytic activity of the enzyme, as these residues could be protected from modification using the inhibitor 1-deoxynojirimycin. 1-Deoxynojirimycin could not prevent inactivation of enzyme treated with N-bromosuccinimide used to modify tryptophan residues. Therefore, the binding mechanism of yeast enzyme contains different amino acid residues compared to its mammalian counterpart.

Catalytically active polypeptides were isolated from endogenous proteolysis and controlled trypsin hydrolysis of the enzyme. A 37 kDa non-glycosylated polypeptide was isolated as the smallest active fragment from both digests, using affinity chromatography with inhibitor-based resins (N-methyl-N'-carboxypentyl-deoxynojirimycin and N'-carboxypentyl-deoxynojirimycin). N-terminal sequencing confirmed that the catalytic domain of the enzyme is located at the C-terminus. The hydrolysis sites were between Arg521 and Thr522 for endogenous proteolysis and between residues Lys524 and Phe525 for the trypsin generated peptide. This 37 kDa polypeptide is 1.9 times more active than the 98 kDa protein when assayed with the synthetic trisaccharide, α-D-Glc-(1,2)-α-D-Glc-(1,3)-α-D-Glc-O-(CH₂)₆COOCH₃, and is not glycosylated.
4.2 INTRODUCTION

The steps involved with assembly and early processing of N-linked glycoproteins in the endoplasmic reticulum (ER) are conserved between lower and higher eukaryotes (Dairaku & Spiro 1997; Gemmill & Trimble 1999). Removal of the distal α-(1,2) glucose residue is initiated by processing α-glucosidase I (EC 3.2.1.106) immediately after transfer of Glc3Man9GlcNAc2 to the protein (Kilker, Jr. et al. 1981). This process is followed by trimming of the two remaining α-(1,3) glucose residues by α-glucosidase II (Grinna & Robbins 1979; Michael & Kornfeld 1980). Glucose residues of the N-linked glycan serve as signals for the protein folding and degradation system in the ER. Therefore α-glucosidase I, in conjunction with α-glucosidase II, plays an integral role in the N-glycoprotein quality control system (Jakob et al. 1998b).

In S. cerevisiae, CWH41 encodes processing α-glucosidase I (Cwh41p) (Romero et al. 1997) which is a member of family 63 of the glycoside hydrolases (Coutinho & Henrissat 1999). The enzymes in this family have been shown to act with inversion of configuration (Palcic et al. 1999). Cwh41p is a type II membrane glycoprotein with a proposed domain orientation comparable to its mammalian counterpart (Jiang et al. 1996; Kalz-Fuller et al. 1995; Shailubhai et al. 1991). Sequence comparison between enzyme orthologs (i.e. human, C. elegans and S. cerevisiae) shows higher identity (34-49%) at the end of the C-terminus domain where the putative catalytic domain is located (Romero et al. 1997). Indeed, proteolysis of membrane-bound mammalian (Bause et al. 1989; Shailubhai et al. 1991) and the soluble yeast α-glucosidase I, as described in Chapter III, liberates catalytically active polypeptide(s) from the luminal domain.

Yeast α-glucosidase I is sensitive to chemical modification with EDAC indicating the importance of carboxylic acid residue(s) for the enzyme activity (Chapter II). Other residues,
including Arg, Cys, and Trp, were reported to be likely participants in the binding site of mammalian \(\alpha\)-glucosidase I based on chemical modification (Pukazhenthi et al. 1993a; Romaniouk & Vijay 1997). Also mutated \(\alpha\)-glucosidase I isolated from a patient with congenital disorder of glycosylation type IIb showed that together, Arg486Thr and Phe652Leu substitutions largely inactivated the enzyme (De Praeter et al. 2000; Volker et al. 2002). Recently, mutations of highly conserved residues, Gly725Arg in the yeast enzyme, and Ser321Phe in enzyme of Chinese hamster ovary cells or the analogous mutation, Ser440Phe in the human enzyme, were also found to impair enzyme activity (Hitt & Wolf 2004; Hong et al. 2004). However, further kinetic and structural studies are required to determine the role of these residues.

It has been previously shown in Chapter II that yeast \(\alpha\)-glucosidase I is sensitive to modification of His and not Cys, distinguishing it from the mammalian enzyme. However, involvement of other residues, such as Arg, Trp and Tyr, in the catalytic activity of yeast \(\alpha\)-glucosidase I has not been reported. Therefore this study was conducted to evaluate the effect of specific chemical modification of Arg, Trp, and Tyr, as well as to provide further information about the role of His in the yeast \(\alpha\)-glucosidase I. Furthermore, the isolation and partial characterization of the active polypeptide released by endogenous proteolysis and controlled trypsin hydrolysis of yeast \(\alpha\)-glucosidase I is reported.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Materials

Diethylpyrocarbonate (DEPC), phenyl glyoxal (PG), N-bromosuccinimide (NBS), and the glucose assay kit (GAGO-20) were obtained from Sigma and tetranitromethane (TNM) was from Aldrich. Sequencing grade-modified trypsin was obtained from Promega,
and N-glycosidase F was the product of Boehringer-Mannheim. DNM-HCl salt was a product of Genzyme. Chemicals used in this study were reagent grade unless otherwise stated.

4.3.2 Yeast strains and growth of transformed yeast overexpressing CWH41

Transformed yeast with CWH41 (Dhanawansa et al., 2002) was used for isolating α-glucosidase I. The transformed yeast cells were cultured in a fermenter according to the method described in Chapter III.

4.3.3 Purification of the soluble form of yeast processing α-glucosidase I

Enzyme was extracted from approximately 40 g of freshly harvested transformed yeast. Purification of the soluble form of α-glucosidase I to 95% and 100%, as determined by Coomassie stained SDS-PAGE, was achieved with the methods described in Chapter II and III.

4.3.4 Enzyme assay

α-Glucosidase I activity was assayed using the synthetic trisaccharide as described in section 2.3.9. The effect of all chemical modification agents and buffers on the coupling enzymes was determined. In cases where the conditions inhibited the coupling enzymes, extensive dialysis in 20 mM sodium phosphate buffer, pH 6.8, was carried out prior to activity determinations.
4.3.5 Site-specific chemical modification of α-glucosidase I

Site specific chemical modification of α-glucosidase I was performed by DEPC, TNM, NBS, and PG for His, Tyr, Trp, and Arg residues, respectively. To achieve the selective modification, optimum conditions for the specific modification were adapted from other studies (Kochhar et al. 1992; Romaniouk & Vijay 1997). Table 4.1 shows the conditions that were used in this study.

4.3.6 Endogenous hydrolysis of 95% purified α-glucosidase I

The 95% purified soluble α-glucosidase I was kept at 4°C for 60 days. The pattern of hydrolysis and α-glucosidase I activity was monitored after 30 and 60 days by SDS-PAGE.

4.3.7 Trypsin hydrolysis of highly purified α-glucosidase I

The pure soluble yeast α-glucosidase I in 100 mM sodium phosphate buffer (pH 6.8) was mixed with sequencing grade modified trypsin (approximate ratio of 10:1) and incubated at 37°C for 150 minutes or 20°C for 20 h. Enzyme without trypsin was incubated under the same conditions. Aliquots were taken to determine the pattern of hydrolysis and activity at various times. α-Glucosidase I activity was measured for the hydrolyzed enzyme and the control sample after trypsin activity was inhibited with PMSF (100 μg/ml).

4.3.8 Isolation of catalytically active polypeptides by CP-DNM-based resins

Catalytically active polypeptides, released by endogenous and trypsin hydrolysis of the 95% and 100% α-glucosidase I, were isolated by methyl-CP-DNM and CP-DNM, respectively. Active polypeptides in 100 mM sodium phosphate buffer containing 0.5 M
NaCl (binding buffer) were applied to a DNM-based resin equilibrated with the same buffer in a batch mode manner. Unbound polypeptides were removed by washing with 15 bed volumes of binding buffer and polypeptides with α-glucosidase I activity were eluted with binding buffer containing 50 mM DNM. Eluted polypeptides were dialyzed overnight against 4 litre of 100 mM sodium phosphate buffer before measuring activity.

4.3.9 Deglycosylation with N-glycosidase F

Pure α-glucosidase I and the 37 kDa active polypeptide were dialyzed in 20 mM sodium phosphate buffer, pH 7.5, containing 100μg/ml PMSF, 0.2% SDS, and 0.5% n-octylglucoside and were heated for 3 min at 95°C. After cooling, N-glycosidase F (~0.4 U) was added and the samples were incubated overnight at 30°C.

4.3.10 Other methods

SDS-PAGE was conducted as described in section 2.3.10. Electro-blotting of the polypeptides was carried out using the Bio-Rad Mini Trans-Blot system onto PVDF membranes as described in section 2.3.7. N-terminal sequencing of blotted polypeptides was performed by the Nucleic Acid Protein Services Unit, Biotechnology Laboratory, University of British Columbia, using standard gas phase Edman chemistry. Protein concentration was determined according to the method of Bradford (1976) as described in section 2.3.20.

4.4 RESULTS

4.4.1 Site-specific modification of α-glucosidase I

Chemical modification using different concentrations of site selective reagents was employed to identify amino acid residues that may participate in the α-glucosidase I catalytic
site. Table 4.1 summarizes the results of different reagents on inhibition of the soluble \(\alpha\)-glucosidase I activity.

4.4.2 Histidine modification with diethylpyrocarbonate

Treatment of \(\alpha\)-glucosidase I with 10 mM DEPC, 25 mM DNM, and the combined treatment of DNM followed by DEPC, reduced \(\alpha\)-glucosidase I activity by 80-90\% (Figure 4.1 A). After dialysis, 78\% of activity was recovered from \(\alpha\)-glucosidase I treated with DNM alone, and 54\% of enzyme activity was recovered from the \(\alpha\)-glucosidase I first protected with DNM and then reacted with DEPC. However, only 13\% of activity could be recovered from the enzyme treated with DEPC alone. DEPC-modified enzyme and unmodified \(\alpha\)-glucosidase I was treated with hydroxylamine (100 mM), which has been shown to restore enzyme activity by decarbethoxylation of the N-carbethoxyimidazole derivative (Christendat & Turnbull 1996; Godavarti et al. 1996). As hydroxylamine was inhibitory to the coupling enzymes used for the activity assay, samples were exhaustively dialyzed prior to assaying for recovery of activity. Hydroxylamine was ineffective in restoring activity in the DEPC-modified enzyme (Figure 4.1 A). The increase in activity of the DNM and DNM-DEPC modified \(\alpha\)-glucosidase I can be attributed to the removal of residual DNM during dialysis. The failure of hydroxylamine to restore activity of the DEPC modified \(\alpha\)-glucosidase I likely is the result of formation of \(N,N'\)-dicarbethoxyhistidine. The formation of this double substituted adduct of His in the presence of excess of DEPC is irreversible (Miles 1977; Roosemont 1978).

Although DEPC reacts preferentially with unprotonated His residues at pH values less than 7, producing the mono- and di-carbethoxyimidazole derivatives with an absorbance near 240-245 nm, it can also react with Tyr residues, resulting in a reduction of absorbance at
280 nm (Miles 1977; Roosemont 1978). Treatment of α-glucosidase I with DEPC increased absorbance at 245 nm with no decrease at 280 nm (Figure 4.1 B), indicating that treatment of α-glucosidase I with DEPC under the described condition was specific for His residue. Therefore, the protection of α-glucosidase I with DNM against inactivation suggests that a critical His residue may be involved in the mechanism of reaction of α-glucosidase I.

4.4.3 Tyrosine modification with tetranitromethane

Specific modification of Tyr residues with TNM proceeds at alkaline pH and produces 3-nitrotyrosine as a final adduct (Sokolovsky et al. 1966). α-Glucosidase I was dialyzed in 25 mM Tris-HCl, pH 8.0, and the effect of different TNM concentrations (1, 5 and 10 mM) on α-glucosidase I was examined (Figure 4.2 A). The highest inhibitory effect was obtained by 10 mM TNM, which reduced α-glucosidase I activity by 90%. However, when α-glucosidase I was reacted first with 25 mM DNM followed by 10 mM TNM, 80% of enzyme activity could be recovered after dialysis (Figure 4.2 B). Although the specific activity of α-glucosidase I dropped approximately 5-fold from its original level due to dialysis into Tris buffer, pH 8.0, DNM was still able to bind to the enzyme active site and protect a critical Tyr residue.

4.4.4 Tryptophan modification with N-bromosuccinimide

NBS at pH less than 7 was shown to be highly selective toward Trp residue modification (Spande and Witkop, 1967). α-Glucosidase I was inhibited by NBS in a concentration-dependent manner (Figure 4.3 A). Enzyme treated with 50 μM NBS lost 96% of its activity, and pre-treatment with 25 mM DNM was not able to prevent enzyme
inactivation (Figure 4.3 B). As well, no activity was recovered after dialysis of NBS modified enzyme (Figure 4.3 B), suggesting that the modification produced a stable adduct.

4.4.5 Arginine modification with phenyl glyoxal

Modification of Arg residues using 20 mM phenylglyoxal (PG) in 100 mM sodium bicarbonate, pH 8.0 inactivated 95% of the enzyme activity. However, with 25 mM DNM alone, only 33% of the unmodified enzyme activity could be inhibited under these conditions. As well, the PG adduct was rather unstable, and 92% of the starting activity was recovered after dialysis with 100 mM sodium bicarbonate, pH 8.0. To rule out the effect of the bicarbonate buffer on enzyme activity, enzyme was treated with 20 mM PG in 100 mM phosphate buffer, pH 6.8, for 1 hour at room temperature. This resulted in only a 49 % loss of enzyme activity.

4.4.6 Isolation of active polypeptide(s) containing the catalytic domain of α-glucosidase I

It was previously shown that the soluble form of α-glucosidase I, purified to approximately 95% homogeneity, retained activity and was endogenously cleaved to several polypeptides after 30 days storage at 4°C (Chapter III). Extending the storage time to 60 days at 4°C (Figure 4.4, lane 2) did not change the pattern of hydrolyzed polypeptides in comparison to 30 days (Figure 3.7). Isolation of two polypeptides from the hydrolyzed enzyme, with the approximate molecular masses of 58 kDa and 37 kDa, was achieved using the inhibitor-based resin N-methyl-N-5'-carboxypentyl deoxynojirimycin (methyl-CP-DNM) (Figure 4.4, lane 3). N-terminal sequencing of the 37 kDa polypeptide band demonstrated that endogenous cleavage occurred between residues Arg521 and Thr522, a potential trypsin
cleavage site. Both peptides were eluted from the resin in low yield (~1.2% of applied activity) and required an extended incubation at 4°C to be generated in an uncontrolled manner by the minor impurities in the enzyme preparation. A controlled hydrolysis using modified trypsin also produced peptides of similar molecular weights (Figure 3.8). Therefore the formation of polypeptides during trypsin hydrolysis, and the activity of α-glucosidase I was monitored at 37°C for 150 minutes (Figure 4.5). Enzymatic activity increased for the first 45 minutes of the hydrolysis, reaching a maximum of 1.4 times the original at 45 minutes (Figure 4.5 A). The reduction of the total activity after 45 minutes incubation at 37°C was likely due to denaturation of active polypeptide(s). As hydrolysis progressed there was an increase in the intensity of bands at 58 and 37 kDa and a corresponding decrease in intensity of the original band at 98 kDa (Figure 4.5 B).

Reducing the temperature of the trypsin hydrolysis to 20°C and extending the time to 20 h produced a mixture of polypeptides in which the 37 kDa peptide was present at a higher concentration (Figure 4.6), and a 1.9-fold increase in activity of the polypeptide mixture was observed. α-Glucosidase I activity of the hydrolyzed enzyme was completely inhibited by 25 mM DNM after 10 min incubation at 37°C. Therefore the increase in activity appears to be associated with cleavage of the intact enzyme to the 37 kDa polypeptide.

To isolate the catalytically active polypeptide from the mixture of hydrolyzed enzyme, CP-DNM resin was used in a batch mode. The 37 kDa polypeptide was eluted from the resin (Figure 4.7, lane 5) with the yield of 8.8%, along with a minor 32 kDa band. N-terminal sequencing of the 37 kDa fragment demonstrated that the cleavage site of this fragment was located between residues Lys524 and Phe525. The theoretical mass of Phe525- Phe833 is 36,026 Da (URL: http://ca.expasy.org/tools/pi_tool.html), which closely matches the mass estimated from SDS-PAGE (Figure 4.7). The 37 kDa active polypeptide
was not an N-glycosylated polypeptide, as the molecular weight remained unchanged after treatment with N-glycosidase F (Figure 4.8).

4.5 DISCUSSION

Processing α-glucosidase I removes the outermost glucose residue from the newly assembled N-glycan. Despite the highly conserved function of this enzyme and overall similarity in domain orientation, it is not known if the orthologs are comparable in binding and catalytic residues.

At the early stage of this research it was noticed that yeast α-glucosidase I is sensitive to modification of His residues (Chapter II). In this study investigation was extended to establish the role of His in more detail. Evidence from this study suggests that a His residue is located near the active site, as DNM could protect against the inactivation of α-glucosidase I activity with DEPC. A critical His residue is also involved in the activity of the plant enzyme which is reported to be sensitive to DEPC modification (Zeng & Elbein 1998). In contrast, the mammalian α-glucosidase I is not as sensitive to DEPC modification (Romaniouk & Vijay 1997; Zeng & Elbein 1998). His residues have not been implicated as acting directly in the catalytic mechanism of glycosidases. However, they have been reported to participate in the binding site and contribute to stabilization of the transition-state of several glycosidases such as β-galactosidase (Escherichia coli) and oligo-1,6-glucosidase (Bacillus cereus) (Huber et al. 2001; Sogaard et al. 1993; Watanabe et al. 2001). Alignment of the α-glucosidase I sequences from S. cerevisiae (Swiss-Prot: P53008), A. thaliana (Swiss-Prot: O64796), and the putative enzyme from Oryza sativa (GenPept Accession: BAB86175) using T-Coffee, Version 2.11 (Notredame et al. 2000) yielded a single conserved His residue in the catalytic region at positions 686, 712 and 734, respectively (Figure 4.9). Interestingly,
alignment of the plant and yeast α-glucosidase I with their mammalian counterparts (H. sapiens Swiss-Prot: Q13724, Mus musculus Swiss-Prot: Q9Z2W5, and Rattus rattus Swiss-Prot: O88941) and the enzyme from C. elegans (Swiss-Prot: Q19426) shows an Ala residue at this location. A second conserved His residue corresponding to His660 in the yeast sequence is observed when all ortholog sequences are aligned. However, the low sensitivity of the mammalian enzymes to DEPC modification makes His660 less likely to be involved in the catalytic activity of yeast and plant enzymes. Further work is required to establish if His686 in yeast α-glucosidase I is the critical residue protected from DEPC modification by DNM.

The specific modification of Tyr residue(s) resulted in approximately a 90% loss of the total enzyme activity. DNM protected against this modification as 80% of the enzyme activity was recovered from the enzyme protected by DNM prior to TNM modification. This suggests that a Tyr residue is likely located at the binding site of the enzyme. A unique Tyr/Glu pair has been found to act as the nucleophile in retaining neuraminidases (family 33) (Ghate & Air 1998). However, as of yet, no inverting enzymes have been shown to utilize Tyr/Glu as a base. Alternatively, aromatic residues can stack on the faces of the sugar residues, affecting substrate orientation (Matsui et al. 1994). Aromatic residues appear to be important for activity of mammalian α-glucosidase I. Mutation of Phe652Leu resulted in inactivation of human α-glucosidase I, with an accompanying loss of binding to an affinity column (Volker et al. 2002). Interestingly, this residue corresponds to Tyr668 in S. cerevisiae, Tyr669 in A. thaliana, and Phe588 in C. elegans α-glucosidase I (Figure 4.10). However, it should be noted that there are several other Tyr residues in the catalytic region of the yeast enzyme that align with Tyr or Phe residues in C. elegans, A. thaliana, and O. sativa and any of these may be involved in the binding site. Rat mammary α-glucosidase I is also
highly sensitive to Tyr modification although neither DNM nor Glc₃Man₂GlcNAc₂ protected this enzyme against TNM inactivation (Romaniouk & Vijay 1997), suggesting a possible structural role in this ortholog.

Modification of Trp residue(s) with NBS also inactivated the enzyme in a concentration dependent manner but DNM was not able to protect α-glucosidase I against inactivation. This suggests that Trp may be important structurally rather than catalytically. Alternatively, DNM may not completely protect the residues of the binding site. The binding site of the enzyme accommodates, and indeed requires, at least three sugar residues as the disaccharide, kojibiose (α-D-Glc-α(1,2)-D-Glc), is not hydrolyzed by the enzyme as it was determined in Chapter II but is an inhibitor (Bause et al. 1986). While it is possible that more than one DNM residue can be accommodated in the active site, as shown for inverting glucoamylase (Harris et al. 1993), not all the residues involved in catalytic activity may be protected by DNM. In contrast, the mammalian enzyme was sensitive to modification of Trp residue and the activity was protected against chemical modification by DNM (Romaniouk & Vijay 1997).

Modification of Arg residues with PG in sodium bicarbonate (pH 8.0) and phosphate buffer (pH 6.8) gave inconclusive results. Under the conditions used, the PG adduct was unstable and DNM could not effectively bind to the enzyme. This indicated that it was not possible to protect the active site at the optimum condition for modification of Arg. Although these results do not completely exclude the possible involvement of an Arg residue at the binding site of the yeast enzyme, it seems that this residue does not play a critical mechanistic role in yeast as it does for its mammalian counterpart (Romaniouk & Vijay 1997).
Together, the results of the chemical modification suggest that the binding motif for the yeast enzyme differs from that of Glu594-Trp602 proposed for the mammalian enzyme (Romaniouk & Vijay 1997). In yeast, the corresponding region, Glu613-Trp621, does not contain His and Tyr residues, which indicates that binding residues are not restricted to this region. Similar to the yeast enzyme, α-glucosidase I from mung bean seedling is sensitive to modification of His and not Cys residues (Zheng and Elbein, 1998), suggesting that the binding motif may differ between lower and higher eukaryotes. Site-directed mutagenesis and detailed kinetic evaluations will be necessary to establish the identity of the critical binding residues.

However, despite these differences, some structural features appear to have been conserved between α-glucosidase I from lower and higher eukaryotes. In this work, similar to other reports for mammalian enzymes (Bause et al. 1989; Shailubhai et al. 1991), proteolysis of the yeast enzyme resulted in progressive hydrolysis to a relatively small resistant polypeptide. This polypeptide, that had significantly higher activity than the intact enzyme, was comprised of approximately one third of the C-terminal protein sequence and was released by both an endogenous hydrolysis and a trypsin proteolysis. Cleavage sites for the endogenous protease, Arg521, and for trypsin, Lys524, were three residues apart suggesting that this region of the 98 kDa soluble Cwh41p may be exposed and susceptible to proteolytic cleavage. These cleavage sites are contained within a region that has no homology with the mammalian enzyme sequences.

Of the five potential N-glycosylation sites for yeast α-glucosidase I, Asn42 and Asn122 have a higher probability for glycosylation (Figure 1.5 A). Two of the less likely glycosylation sites, Asn787 and Asn805, are included in the 37 kDa active polypeptide, and it was shown here that these are not N-glycosylated. In contrast, a 39 kDa catalytically active
polypeptide isolated from rat $\alpha$-glucosidase I is predicted to be $N$-glycosylated at Asn654, the only predicted $N$-glycosylation site of mammalian (Figure 1.5 B). However, deglycosylation of rat mammary gland $\alpha$-glucosidase I does not drastically decrease enzymatic activity (Shailubhai et al., 1991). This suggests that the $N$-linked oligosaccharide is not required for the activity of the catalytic domain of the yeast or mammalian enzyme. However, it is still not clear if $N$-glycosylation of $\alpha$-glucosidase I orthologs is necessary for the polypeptide to acquire the proper conformation during biosynthesis.

Processing $\alpha$-glucosidase I in all orthologs hydrolyze substrate with net inversion of configuration (Palcic et al. 1999). Regardless of the overall similarity in mechanism and domain structure, there is discrepancy between potential binding residues of orthologs, with plant and yeast exhibiting greater similarity to each other than to mammalian forms of the enzyme.

4.6 FUTURE STUDIES

The results of this study indicated that a 37 kDa C-terminal domain fragment released from the 98 kDa $\alpha$-glucosidase I, is catalytically active and resistant to proteolysis. Therefore it is important to establish if the 37 kDa fragment can be expressed as a catalytically active polypeptide. This will allow further mechanistic studies to focus on this critical region which will provide better insight about the structural-functional relationship between the domains of enzyme and ultimately a better understanding about the mechanism and structure of family 63 glycosidases.

In addition, based on studies with the mammalian enzyme (Shailubhai et al. 1991) and this study, it is suggested that the $N$-glycosylation of $\alpha$-glucosidase I is not important for
the function of the orthologs. However, this speculation should be examined by expression of the non-glycosylated mutant forms of the enzyme.
Table 4.1. Maximum inhibitory effect after site-specific chemical modification of selected amino acids of *S. cerevisiae* processing α-glucosidase I.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Reagent *</th>
<th>Maximum inhibition (%)</th>
<th>Modification condition (concentration of reagent, buffer, pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>His</td>
<td>DEPC</td>
<td>90</td>
<td>10 mM, 20 mM sodium phosphate, pH 6.8</td>
</tr>
<tr>
<td>Trp</td>
<td>NBS</td>
<td>96</td>
<td>50 μM, 20 mM sodium phosphate, pH 6.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>TNM</td>
<td>90</td>
<td>10 mM, 25 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>Arg</td>
<td>PG</td>
<td>95</td>
<td>20 mM, 100 mM sodium bicarbonate, pH 8.0</td>
</tr>
</tbody>
</table>

* All modifications were performed at room temperature for 1 hour.
Figure 4.1. α-Glucosidase I activity is protected by DNM against modification with DEPC. (A) Aliquots of enzyme (247.5 µl, 16 U) in 20 mM sodium phosphate buffer pH 6.8 were treated with (a) 10 mM DEPC, (b) 25 mM DNM, or (c) 25 mM DNM followed by 10 mM DEPC for 1 hour at room temperature. Remaining activity was measured before (■) and after (□) overnight dialysis against 4 litre of 20 mM sodium phosphate buffer pH 6.8. Subsequently all samples were treated with 100 mM hydroxylamine for 20 min at room temperature and enzyme activity measured after overnight dialysis (□). Results are presented as remaining activity compared to the appropriate control for two independent experiments. Error bars represent the range of the two measurements. (B) Specific reaction of DEPC with His residue. Changes in absorbance (ABS) of α-glucosidase I treated with 10 mM DEPC (II) was scanned (238-288 nm) for 1 h incubation at room temperature and compared to untreated control enzyme (I).
Figure 4.2. α-Glucosidase I modification with TNM. (A) α-glucosidase I is inactivated by TNM in a concentration dependent manner. Aliquots of enzyme (20 μl, 0.75 U) in 25 mM Tris-HCl pH 8.0 were incubated for 1 h at room temperature with TNM in ethanol (0, 1, 5, and 10 mM). Results are presented as remaining activity compared to the control. (B) α-glucosidase I activity is protected by DNM against modification with TNM. Aliquots of enzyme (58 μl, 2.14 U) in 25 mM Tris-HCl pH 8.0 were treated with (a) 10mM TNM, (b) 25 mM DNM, and (c) 25 mM DNM, then 10mM TNM for 1 hour at room temperature. Remaining activity was measured before (■) and after (□) overnight dialysis against 3 litre of 25 mM Tris-HCl pH 8.0 at 4°C. Results are presented as remaining activity compared to the appropriate control.
Figure 4.3. α-Glucosidase I modification with NBS. (A) α-Glucosidase I is inactivated by NBS in a concentration dependent manner. Enzyme (90 µl, 3.6 U) in 20 mM sodium phosphate buffer pH 6.8 was incubated for 1 hour incubation at room temperature with NBS (0, 10, and 50 µM). (B) α-Glucosidase I activity is not protected by DNM against modification with NBS. Aliquots of enzyme (90µl, 3.8 U) in 20 mM sodium phosphate buffer pH 6.8 were treated with (a) 50 µM NBS, (b) 25 mM DNM, and (c) 25 mM DNM, then 50 mM NBS for 1 hour at room temperature. Remaining activity was measured before (■) and after (□) overnight dialysis against 4 litre of 20 mM sodium phosphate buffer pH 6.8 at 4°C. Results are presented as the remaining activity compared to the appropriate control for two independent experiments. Error bars represent the range of the two measurements.
Figure 4.4. Endogenous hydrolysis of partially purified $\alpha$-glucosidase I produced catalytically active polypeptides, isolated by methyl-CP-DNM resin. A 1.4 ml aliquot of 95% purified $\alpha$-glucosidase I (lane 1) was kept at 4°C for 60 days. The mixture of catalytically active polypeptides (lane 2) was applied to the 500 µl of the methyl-CP-DNM resin and mixed overnight at 4°C. Unbound proteins removed by washing of the resin with 15 bed volume of resin. Active polypeptides were eluted from the resin with 50 mM DNM in binding buffer (lane 3). Samples were subjected to SDS-PAGE 8-25% (PhastGel), lane 1 and 2 were stained by Coomassie blue while lane 3 was silver stained.
Figure 4.5. Trypsin hydrolysis of pure α-glucosidase I at 37°C produced catalytically active polypeptides. Pure α-glucosidase I (22.5 μg) was incubated with 2.5 μg of modified trypsin and incubated at 37°C in a final volume of 1 ml for 150 minutes. Samples were withdrawn at the noted time intervals. A 40 μl aliquot was mixed with the PMSF (100 μg/ml). (A) The percentage of remaining activity of a 4 μl aliquot at different time intervals as compared to the total activity of enzyme at 0 min (control). (B) The remaining sample (36 μl) was mixed with SDS-PAGE sample buffer, boiled for 5 min, subjected to the 12.5 % SDS-PAGE, and was stained by Coomassie blue.
Figure 4.6. Trypsin hydrolysis of pure α-glucosidase I at 20°C produced a 37 kDa catalytically active polypeptide. Pure α-glucosidase I (1 ml, 24.7 μg) was mixed with the modified trypsin (3 μg) and kept at 20°C for 20 h. Enzyme without trypsin was incubated under the same conditions (control). An aliquot of the hydrolyzed enzyme and control were mixed with SDS-PAGE sample buffer, boiled for 5 min, subjected to 15% SDS-PAGE, and Coomassie stained. Enzyme only (control, lane 1), hydrolyzed α-glucosidase I (lane 2), and molecular weight standards (lane 3). The arrow shows the position of modified trypsin on the gel. The α-glucosidase I activity was measured for the hydrolyzed enzyme and control after trypsin activity was inhibited with PMSF (100 μg/ml). The values above the figure represent the ratio of total activity of the hydrolyzed sample without purification to control.
Figure 4.7. The 37 kDa catalytically active polypeptide was isolated by CP-DNM chromatography from trypsin hydrolyzed α-glucosidase I. Hydrolyzed enzyme (800 µl), as described in Figure 4.6, was applied to the CP-DNM resin (400 µl). Absorbed activity was eluted with 50 mM DNM in binding buffer. Eluted polypeptides were dialyzed and activity was measured. The values given above the figure represent total activity (U) measured at each corresponding stage. Modified trypsin (lane 2) and hydrolyzed α-glucosidase I (lane 4), were subjected to 15% SDS-PAGE. Eluted polypeptides (lane 5) from CP-DNM resin were blotted on the PVDF membrane. Lanes 1, 3 and 6 show molecular weight standards. Gel and PVDF were stained by Coomassie blue. The arrow shows the band that was sequenced.
Figure 4.8. The 37 kDa active polypeptide containing the catalytic domain of α-glucosidase I is not N-glycosylated. The 37 kDa polypeptide (A) and α-glucosidase I (B) treated with N-glycosidase F (+) or untreated (-), were subjected to SDS-PAGE (12.5%) and Coomassie stained. The 37 kDa polypeptide was generated as described in Figure 4.6.
<table>
<thead>
<tr>
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<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>A. thaliana</td>
<td>LKLV-PH712LGYVS</td>
</tr>
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</tr>
<tr>
<td>C. elegans</td>
<td>TL--VSDA636FGYNN</td>
</tr>
</tbody>
</table>

Figure 4.9. A single conserved histidine residue in the 37 kDa catalytically active polypeptide of S. cerevisiae α-glucosidase I aligns with a histidine in A. thaliana and O. sativa, and an alanine residue in mammalian and C. elegans orthologs. Sequence alignment was carried out using T-Coffee, Version 2.11 (URL: http://igs-server.cnrs-mrs.fr/Tcoffee).
Figure 4.10. Alignment of conserved tyrosine or phenylalanine residues of α-glucosidase I orthologs with human Phe652, a residue critical for activity of the human enzyme (Volker et al. 2002). Sequence alignment was carried out using T-Coffee, Version 2.11.
CHAPTER V

PART 4. FUNCTIONAL DOMAINS AND IMPORTANT CARBOXYLIC ACID RESIDUES (E613 and D617) OF α-GLUCOSIDASE I
Chapter V

5.1 ABSTRACT

α-Glucosidase I, Cwh41p, is a type II membrane glycoprotein residing in the endoplasmic reticulum involved in the N-glycosylation pathway. However, a soluble form of the enzyme is released by endogenous cleavage between (A24-T25) of the transmembrane domain. In addition, it was established that the catalytic domain, located within F525-F833 region, is resistant to hydrolysis. In this study functional expression of truncated forms of Cwh41p, namely Cwht1p (E35- F833), Cwht2p (R320-F833), and Cwht3p (M526-F833) fused with 6×His residues at the C-terminal, was investigated. Immunoblot analysis and α-glucosidase I assay revealed that only Cwht1p was expressed. Activity of yeast expressing CWHT1 was 1400 U/g of biomass, approximately 4-fold higher than yeast overexpressing CWH41. Cwht1p was purified as a 94 kDa non-glycosylated polypeptide with a specific activity (3600 U/mg of protein) comparable to that of the soluble α-glucosidase I (3000 U/mg of protein). Immunoblot analysis indicated that Cwht1p was not associated with the membrane. These results suggest that the M1-128 region of Cwh41p likely carries an ER-targeting signal sequence and is not important for protein folding. These findings also established that the active conformation of enzyme is not dependent on protein glycosylation.

Alignment of α-glucosidase I orthologs indicated that there are six highly conserved carboxylic acid residues contained within the catalytic region (F525-F833) of yeast α-glucosidase I; of these E613 and D617 are situated at the corresponding proposed binding motif of the mammalian enzyme. Substitution of E580 and D584 of Cwht1p (corresponding to E613 and D617 of Cwh41p) with Ala resulted in undetectable α-glucosidase I activity with the synthetic trisaccharide substrate. Moreover, mutants were expressed at significantly lower concentrations than Cwht1p. These findings suggest that E613 and E617 play an
important role in yeast α-glucosidase I activity, although it is not established if this is structural or catalytic in nature.

5.2 INTRODUCTION

Mannosyl-oligosaccharide glucosidase or processing α-glucosidase I (EC. 3.2.1.106) belongs to the family 63 of glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/). Despite the key role of α-glucosidase I in the N-glycosylation pathway it has remained one of the least characterized glycosidases in the ER due to difficulties with overexpression, insufficient quantity of pure enzyme, the unique substrate specificity, and instability especially in higher eukaryotes (Romaniouk et al. 2004). Furthermore, family 63 exclusively contains processing α-glucosidase I indicating that this enzyme does not have significant structural similarity with other known glycosidases. Therefore, it is difficult to infer mechanistic information about α-glucosidase I from other families of glycosyl hydrolases.

α-Glucosidase I orthologs exhibit a conserved function in the ER and possess comparable structural features (Jiang et al. 1996; Kalz-Fuller et al. 1995; Romero et al. 1997). It has been reported that orthologs are type II membrane-bound enzymes residing in the ER and containing a C-terminal catalytic region resistant to proteolysis (Bause et al. 1989; Faridmoayer & Scaman 2005; Shailubhai et al. 1991). Also, orthologs have demonstrated some comparable biochemical properties i.e. neutral pH optimum and sensitivity to similar inhibitors (Herscovics 1999b). In contrast, several discrepancies such as dissimilarities in potential binding residues (Faridmoayer & Scaman 2005; Romaniouk & Vijay 1997; Zeng & Elbein 1998) and different affinity toward natural oligosaccharide truncated at mannose residues have been noted between the enzyme from lower and higher eukaryotes (Grinna & Robbins 1980; Saunier et al. 1982; Zeng & Elbein 1998).
Yeast α-glucosidase I (Cwh41p), like its mammalian counterpart, is a type II integral membrane N-glycoprotein (833 residues) and is encoded by CWH41 (Romero et al. 1997). Cwh41p (Swiss-Prot: P53008) is predicted to have a short cytosolic N-terminus domain (M1-K10), a large C-terminus domain (S29-F833) extended toward the lumen of the ER and a hydrophobic transmembrane domain (T11-I28) in between (Figure 5.1) (Jiang et al. 1996). Previously it was shown that endogenous cleavage of the predicted signal peptide cleavage site (Ala24 and Thr25) at the suggested transmembrane region of Cwh41p (Figure 5.1) liberated the soluble form of α-glucosidase I activity (Chapter II). Furthermore, a mixture of catalytically active fragments, 59 and 37 kDa, were released from the soluble α-glucosidase I during endogenous hydrolysis or trypsin proteolysis of the enzyme (Chapter III). The 37 kDa polypeptide was isolated as the smallest non-glycosylated catalytically active fragment from both mixtures. In addition, it was established that the putative catalytic domain of yeast enzyme is located within the F525-F833 region (Chapter IV).

This study was focused on the functional relationship of different domains of α-glucosidase I. The effect of deleting different regions of the enzyme on expression, catalytic activity, and glycosylation was determined. Therefore, truncated forms of CWH41 gene encoding E35-F833 (CWHT1), R320-F833 (CWHT2), and M526-F833 (CWHT3) fused with C-6×His tag were constructed, and the expressed polypeptide was purified and characterized.

It was reported that α-glucosidase I from yeast and mammalian sources cleaves the α-(1,2) glycosidic bond of the synthetic substrate, α-D-Glc-(1,2)-α-D-Glc-(1,3)-α-D-Glc-O-(CH₂)₈COOCH₃ through the net inversion of configuration (Palcic et al. 1999). This process is commonly catalyzed by a pair of carboxylic acid residues (Koshland 1953) where one carboxylic acid acts as a general acid and the other as a general base (Rye & Withers 2000). It was shown that yeast α-glucosidase I activity is sensitive to EDAC, the specific reagent for
modifying carboxylic acid residues (Chapter II). Also, it was reported that catalytic activity of the 37 kDa fragment (F525-F833) was inhibited by DNM, an α-glucosidase I inhibitor (Chapter IV). Collectively these findings suggest that at least one exposed catalytically active carboxylic acid residue resides within the catalytic region F525-F833. Sequence homology between orthologs of α-glucosidase I revealed that six highly conserved carboxylic acid residues (D601, D602, E613, D617, D670, and E804 of Cwh41p) are situated within the catalytic region (Figure 5.2). Furthermore E613 and D617 are located at the proposed binding motif of rat α-glucosidase I (E592 RHLDLRCW600) (Romaniouk & Vijay 1997), which corresponds to Glu613-Trp621 of yeast α-glucosidase I sequence. Therefore, the function of these residues in yeast α-glucosidase I were investigated by substitution of carboxylic residues with Ala. Mutated enzymes were isolated and α-glucosidase I activity was determined. The results of this study provide better insight into the function and relationship of the different domains of yeast α-glucosidase I. As well, it provided preliminary information about the importance of two highly conserved carboxylic acid residues (E613 and D617) residing in the putative catalytic region of yeast α-glucosidase I activity.

5.3 MATERIALS AND METHODS

5.3.1 Materials

All restriction enzymes (i.e. BglII, EcoRI, DpnI, XbaI and XhoI), rapid DNA ligation kit, bacterial transformation kit (TransformAid), and DNA ladder (MassRuler) were obtained from Fermentas. Polymerase chain reaction (PCR) purification, DNA gel extraction, and plasmid purification kits were purchased from Qiagen. Low melting point agarose (Ultrapure) and salmon sperm DNA were products of Gibco-BRL. His-linker
oligonucleotides, PCR, and mutagenic primers were obtained from Qiagen. Oligonucleotides for DNA sequencing were synthesized at the Nucleic Acids and Protein Service facility (NAPS), University of British Columbia. dNTPs were purchased from Amersham-Biosciences. MilliQ ultra pure water was used for all of the steps involved in DNA preparation and manipulation. All buffer chemicals and other reagents were obtained from Sigma, unless otherwise noted.

5.3.2 Microbial strains and media

*E. coli* DH5α (α endA1, hsdR17, supE44, thi-1 1', recA1, gyrA96, relA1, lacU169 [F80d lac ZDM15]) was obtained from the Wine Research Center, University of British Columbia and *Saccharomyces cerevisiae* AH22 (MATα, Leu2-3, leu2-112, his4-519, can1, cir+J) was from ATCC. Transformed and non-transformed *E. coli* strains were grown in Luria-Bertani (LB) Broth with or without 100 μg/ml of ampicillin, respectively. *S. cerevisiae* AH22 was grown in YPD (1% yeast extract (Difco) + 2% Bacto-peptone (Difco) + 2% glucose). Recombinant yeast strains were grown in minimal medium plus 50 mg/l L-histidine as described previously (Chapter II).

5.3.3 Bacterial and yeast transformation

Preparation of competent cells and transformation of plasmids into *E. coli* were carried out using TransformAid kit (Fermentas). Yeast transformation was performed using lithium acetate based method adapted from Gietz and co-workers (Gietz et al. 1992; Schiestl & Gietz 1989). Details of the yeast transformation protocol are described in Appendix 1.
5.3.4 Plasmids

pHVX2, a shuttle vector containing phosphoglycerate kinase gene (PGK1), a constitutive promoter (Volschenk et al. 1997), was used in this study. pHVX2 was kindly provided by Dr. Hennie J. J. van Vuuren, Wine Research Center, University of British Columbia. pRAN1, a pHVX2 vector carrying CWH41 gene (Dhanawansa et al. 2002) was used as the template for construction of truncated fragments by PCR.

5.3.5 Construction of pHVX2-His

pHVX2 was modified to create a new shuttle vector, pHVX2-His which expresses protein with a 6×His at the C-terminus. Two complementary 5’-phosphorylated strands of oligonucleotide in the cut forms with BglII (5’end) and XhoI (3’end), encoding six His residues (His-linker), were designed (Table 5.1). For annealing oligonucleotides, approximately 32.5 pmol of oligonucleotides in 10 mM Tris, pH 7.5 were transferred into a sterile PCR tube, the volume was adjusted to 15 µl with water, and 1.5 µl of annealing buffer (100 µM Tris, pH 7.5, 100 mM MgCl₂, 10 mM DTT) was added. The mixture was heated to 95°C for 5 min in a thermal cycler (Perkin-Elmer, DNA Thermal cycler 480) and cooled slowly until it reached room temperature. Water (6.5 µl) and 2.5 µl of annealing buffer were transferred to a microfuge tube and 1 µl of annealing reaction was added and mixed. pHVX2 was double digested with BglII and XhoI and the cut form of pHVX2 was purified with the gel extraction kit. Approximately 50 ng of purified double digested pHVX2 (~16-18µl) was mixed with 1µl of diluted annealing mixture in a PCR tube. Ligation buffer and T4 DNA ligase was added to the mixture according to the manufacturer’s protocol and incubated overnight at 16°C. The newly formed shuttle vector, pHVX2-His with EcoRI and BglII, between the PGK1 promoter (PGK1_p) and terminator (PGK1_t) sequence, was used for
cloning (Figure 5.3). pHVX2-His was transformed into *E. coli* DH5-α and transferred onto an LB plate with 100 μg/ml ampicillin. pHVX2-His was purified from transformed *E. coli* using a plasmid purification kit and the presence of the His-linker region was verified by DNA sequencing. Purified pHVX2-His without an insert was transformed into *S. cerevisiae* AH22 as the control transformed strain.

### 5.3.6 Construction and subcloning of truncated forms of CWH41

Truncated forms of *CWH41*, *CWHT1* (2400 bp), *CWHT2* (1542 bp), and *CWHT3* (924 bp), were amplified by PCR (Figure 5.4). Upstream primers were synthesized with an *EcoRl* restriction site at the 5' end and down stream primers with a *BglII* site at the 5' end (Table 5.1). PCR reaction mixture was comprised of 10 ng of pRAN1 as a template, 200 μM of each dNTPs, 600 nM of each primer, and 25 μl of 10×PCR buffer (supplied by manufacturer). The volume was adjusted to 50 μl with water and 2.5 U of high fidelity *Pwo* DNA polymerase was added (Roche Diagnostics Gmbh). The thermal cycles used for amplification of truncated forms of *CWH41* were designed according to manufacturer’s protocol. The size of PCR products were verified by agarose gel electrophoresis stained with ethidium bromide (Figure 5.4). Amplified DNAs were purified using the PCR purification kit and were cut with *EcoRl* and *BglII*. Cut forms of *CWH(1-3)* were purified by a gel extraction kit. pHVX2-His was digested with the same restriction enzymes and purified by a gel extraction kit. Ligation of *CWH(1-3)* with the cut pHVX2-His was carried out overnight at 16°C using a DNA ligation kit. Newly formed plasmids, namely pCWHT1, pCWHT2, and pCWHT3, were transformed into *E. coli* DH5-α. Presence of inserts was verified by restriction site analysis and DNA sequencing. Purified pCWHT(1-3) were transformed into *S. cerevisiae* AH22 according to the method described in Appendix 1.
5.3.7 Extraction, isolation, and purification of Cwht(1-3)p

Yeast cells, transformed with pCWHT(1-3) were grown and harvested according to the procedure described in Chapter II. All isolation and purification steps were carried out at 4°C. PMSF (5 mM) was added to the protein fractions that were pooled after each step. The soluble fraction of protein was extracted from the transformed yeast cells according to the method described in section 2.3.4. α-Glucosidase I activity was measured in the microsomal free homogenate after isolation by ultracentrifugation for 1 h at 100,000 ×g. Resultant soluble proteins were then precipitated with 70% saturated ammonium sulfate and the precipitated fractions were solubilized in 20 mM sodium phosphate, pH 6.8.

A single step isolation of the C-6×His tagged polypeptides was carried out using an immobilized metal affinity chromatography (IMAC) column (1 ml His-Trap HP; Amersham-Biosciences). Protein solutions were dialyzed for 4 h in 20 mM sodium phosphate buffer, pH 7.4, containing 10 mM imidazole and 0.5 M NaCl (binding buffer) and applied to the column equilibrated previously with binding buffer, at a flow rate of 0.5 ml/min. Protein fractions were eluted from the column by a step gradient concentration of imidazole (20, 40, 60, 100, 300, and 500 mM) in 20 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl, and fractions of 1 ml were collected. Protein peaks were pooled separately and dialyzed for 4-6 h in 20 mM sodium phosphate buffer, pH 6.8, before measuring α-glucosidase I activity. For active fragments, fractions with higher purity were obtained using anion-exchange chromatography as described in Chapter III (section 3.3.4) following IMAC.

5.3.8 Antibodies and immunoblot analysis

Immunoblot analysis was conducted to detect expression of Cwht(1-3)p. Proteins from different recombinant yeasts were transferred onto PVDF membrane electrophoretically
using the Mini Trans-Blot system (Bio-Rad). Electro-blotting was conducted at 100 V for 1 h in transfer buffer (25 mM Tris base, 192 mM glycine, 20 % methanol and 0.025% SDS). Immunoblotting was then carried out using rabbit anti-yeast α-glucosidase I polyclonal antibody (Appendix 2) and/or mouse anti 6×His-tag monoclonal antibody (Novagen) according to the manufacturer's procedure (Novagen). Detection was carried out using peroxidase conjugated goat anti-mouse polyclonal antibody (BD Biosciences) or peroxidase conjugated goat anti-rabbit polyclonal antibody (Sigma), and 4-chloro-1-naphthol (Sigma) as the substrate.

### 5.3.9 Detection of glycoproteins

A combined enzymatic-chemical approach was employed for identifying glycoproteins. Aliquots of proteins were deglycosylated with N-glycosidase F as described previously in section 2.3.8. Thereafter, samples were dialyzed in 20 mM sodium phosphate buffer, pH 6.8 with a centrifugal filter device (Ultrafree-0.5, Millipore). Ice cold protein solutions (~ 0.5 mg/ml) were then mixed with sodium periodate (NaIO₄) at a final concentration of 10 mM to oxidize the vicinal hydroxyl groups of the glycan. The reaction was carried out for 30 min at 4°C, then the solution was dialyzed in 20 mM sodium phosphate buffer, pH 6.8, using a centrifugal filter device (Ultrafree-0.5, Millipore). Subsequently, dialyzed samples were mixed with biotin hydrazide (Sigma) at a final concentration of 2 mg/ml, and incubated for 2 h at room temperature. Figure 5.5 shows the schematic diagram of the reaction. Samples were run on SDS-PAGE and electroblotted onto PVDF membrane as described in previous section. Biotinylated glycoprotein was then detected by reaction with peroxidase conjugated IgG fraction of anti-biotin from goat (Rockland) on the PVDF membranes, according to the manufacturer's procedure.
5.3.10 Site-directed mutagenesis

Site directed mutagenesis of E580 and D584 of Cwt1p, corresponding to E613 and D617 of Cwh41p, to Ala was carried out using the method developed by Stratagene. Two complementary oligonucleotides containing the mismatch nucleotide at the position of interest, flanked by an unmodified nucleotide sequence were synthesized (Table 5.1). The PCR reaction was comprised of 50 ng of pure ~9.9 kb pCWHT1 (parental plasmid), 125 ng of mutagenic primers, 0.25 mM (final concentration) dNTPs, and 5 µl of 10×PCR buffer (supplied by manufacturer); the final volume was adjusted to 50 µl with water before adding 2.5 U (1 µl) of pfuUltra high-fidelity DNA polymerase (Stratagene). PCR thermal cycling was initiated with a denaturing cycle (1 min at 95°C) and was followed by seventeen cycles (50 s at 95 °C, 55 s at 56 °C, and 10 min at 68 °C). Amplification was checked by electrophoresis of 10 µl of the product on an agarose gel. The remaining PCR products were treated with 15 U of DpnI and incubated for 3-6 h at 37°C to digest the methylated parental plasmid (E. coli DH5-α is dam+, which expresses methylated plasmid i.e. pRAN1). Mutated plasmids were purified by a PCR purification kit and transformed into E. coli DH5-α. pE580A and pD584A were purified from transformed E. coli by a plasmid purification kit and mutations were verified by DNA sequencing of the insert. Purified mutated plasmids were transformed into S. cerevisiae AH22 as described in Appendix 1.

5.3.11 Extraction, isolation, and detection of mutated proteins

Extraction of proteins was carried out using the method described earlier. Single step isolation of 6×His-tagged proteins was performed using IMAC. Samples after IMAC were dialyzed for 6-8 h in 20 mM sodium phosphate buffer (pH 6.8). α-Glucosidase I activity and aryl α-glucosidase activity were measured. The protein after IMAC was further
purified by chromatography on the FPLC MonoQ HR 5/5. Expression of mutated proteins was detected by immunoblotting using an anti 6×His monoclonal antibody.

5.3.12 Enzyme assay

α-Glucosidase I activity and aryl α-glucosidase activity were measured using the synthetic trisaccharide α-D-Glc-(1,2)-α-D-Glc-(1,3)-α-D-Glc-O-(CH₂)₆COOCH₃, and p-nitrophenyl α-D-glucopyranoside, respectively, as described in section 3.3.6. The trisaccharide was a kind gift of Dr. M. Palcic, Department of Chemistry, University of Alberta.

5.3.13 Other methods

Protein concentration was measured as described previously (section 2.3.10). SDS-PAGE was carried out according to the method of Laemmli (1970) (section 2.3.10). DNA samples were separated in agarose gel (0.8% w/v) for 1.5-2 h at 100 V with a TBE buffer (45 mM Tris-borate plus 2 mM EDTA). The gel was stained with ethidium bromide (0.5 µg/ml) and photographed under UV light. A DNA ladder was run in parallel with DNA samples. DNA sequencing was performed at the Nucleic Acids Protein Services unit, University of British Columbia.

5.4 RESULTS

5.4.1 Growth of recombinant yeast strains

Transformed yeast strains with pHVX2-His (control plasmid without insert) and pCWHT(1-3) did not show any discernable phenotype but had a lower growth rate than the yeast transformed with pRAN1 reported earlier. Turbidity (OD₆₀₀) of culture after 18 h
incubation at 28°C with shaking at 300 rpm was 1.8±0.4 which was approximately half of that obtained from the transformed yeast with pRAN1. Therefore, the culture was grown under the same condition until it reached an OD$_{600}$ of 4; at this turbidity, the average yield was 6.9±0.6 g of wet biomass per litre of culture.

5.4.2 DNA sequencing of pCWHT(1-3)

DNA sequencing of the pCWHT(1-3) confirmed that all inserts (CWHT1, CWHT2, and CWHT3) were in frame. Also, sequencing confirmed that all sequences had a start codon (ATG) after the EcoRI site, followed by a truncated sequence of CWH41, a BglII site linking the DNA insert to the His-linker sequence and the stop codon (TGA) at the end (Figure 5.6).

5.4.3 Determination of α-glucosidase I active yeast strains

α-Glucosidase I activity was determined in the cell extracts of transformed yeast strains after removing microsomes (Table 5.2). Total activity of the recombinant yeast strain expressing CWHT1 was 1400 U per g of wet biomass; approximately 4-fold higher than the yeast expressing CWH41 (300 U per g of wet biomass). Other transformed yeast strains (CWHT2 and CWHT3) possessed negligible α-glucosidase I activity towards the synthetic substrate (Table 5.2), indicated that only Cwht1p was catalytically active. Therefore, the other two truncated forms, Cwht2p and Cwht3p, either did not express or were not functional.

5.4.4 Detection and isolation of truncated fragments (Cwht(1-3)p)

Protein isolates, after 70% ammonium sulfate precipitation, were dialyzed in binding buffer and applied to the His-Trap column. Elution of proteins was carried out using
the step gradient concentration of imidazole (20, 40, 60, 100, 300, and 500 mM), as shown in Figure 5.7 A. SDS-PAGE analyses of IMAC fractions revealed that only the 100 mM imidazole fraction of yeast expressing CWHT1 had different proteins compared to the other 100 mM imidazole fractions (Figure 5.7 B, lanes 4). Furthermore, the 100 mM imidazole fraction of yeast expressing Cwhtlp (Figure 5.7 II) was found to be the only fraction that exhibited α-glucosidase I activity. Western blot analyses of cell homogenates with anti-yeast α-glucosidase I antibody (Figure 5.8 B) and anti-His antibody (Figure 5.8 C) detected Cwhtlp, while Cwht2p and Cwht3p were not detected. Moreover, immunoblot analysis of the microsomal proteins (Figure 5.8 A, lane 2) of yeast expressing CWHT1 did not detect α-glucosidase I using polyclonal antibody (Figure 5.8 B, lane 2) or anti-His monoclonal antibody (Figure 5.8 C, lane 2).

5.4.5 Purification of Cwhtlp

His-Trap was not able to purify the Cwhtlp, as the active fraction was not homogeneous on SDS-PAGE (Figure 5.7 B, lane 4). Purification of Cwhtlp to a higher homogeneity was achieved by a combination of ammonium sulfate precipitation (70%), Toyopearl DEAE, FPLC MonoQ HR5/5 (Figure 5.9 A), and IMAC chromatographies (Figure 5.9 B). Table 5.3 shows the purification results. The yield of purified α-glucosidase I was 12%, or approximately 45 μg of Cwhtlp per g of wet biomass. The molecular mass of the purified Cwhtlp subunit appeared to be 94 kDa on SDS-PAGE (Figure 5.9 C).

5.4.6 Cwhtlp is not glycosylated

Purified Cwhtlp as well as the soluble α-glucosidase I (control) were treated with N-glycosidase F. The molecular mass of the soluble α-glucosidase I treated with N-glycosidase
F was reduced (Figure 5.10 A, lane 1 and 2) while no change was observed for Cwht1p (Figure 5.10 A, lane 3 and 4). Subsequently, all samples (treated and untreated with N-glycosidase F) were subjected to chemical modification with sodium periodate and biotin hydrazide. Western blot did not detect any of the Cwht1p samples (Figure 5.10 B, lane 3 and 4) while the soluble α-glucosidase I was found to be biotinylated (Figure 5.10 B, lane 1).

5.4.7 Releasing catalytically active polypeptide(s) by hydrolysis of Cwht1p

Overnight hydrolysis of pure Cwht1p at 20°C with trypsin released a mixture of catalytically active polypeptide(s) (Figure 5.11). α-Glucosidase I activity of Cwht1p hydrolysates was determined to be 1.4-fold higher than Cwht1p. The increase of α-glucosidase I activity has also been reported for endogenous and trypsin hydrolysis of the soluble α-glucosidase I (Chapters III and IV). The polypeptide bands appeared to have molecular masses of 54, 38, 29, and 24 kDa in which the 38 kDa band had the highest intensity, based on SDS-PAGE analysis (Figure 5.11, lane 2).

5.4.8 DNA sequencing of pE580A and pD584A

The Glu580 (GAA) and Asp584 (GAC) codons of Cwht1p, corresponding to E613 and D617 of Cwh41p (Figure 5.12 a), were mutated to an Ala codon (GCA or GCC) by substitution of the middle nucleotide of the carboxylic acid codon from A to C. DNA sequencing of pE580A and pD584A verified codon substitutions at positions of 580 (Figure 5.12 b) and 584 (Figure 5.12 c) of Cwht1p.
5.4.9 Isolation, detection, and purification of Cwht1p mutants (E580A and D584A)

Three colonies from yeasts transformed with pE580A and pD584A were selected randomly. Cultures were grown for 25 h at 28°C with shaking at 300 rpm. The average turbidity (OD$_{600}$) of cultures was 3.3±0.5 and 3.5±0.6 for pE580A and pD584A yeast strains, respectively. Approximately 1.4 g and 1.3 g of wet biomass were harvested from 190 ml of yeast pE580A and pD584A cultures, respectively. Extraction of proteins was carried out separately using the method described in section 2.3.4. Proteins were isolated using a single step IMAC with an imidazole step gradient elution (Figure 5.13 A and B). The 100 mM imidazole IMAC fraction had multiple protein bands, including the protein of interest (Figure 5.13 C). According to the SDS-PAGE profile, the colonies with higher expression levels were selected for the next experiments.

Colonies with potentially higher expression level were grown in 1 litre of media and protein was extracted from approximately 7 g of wet biomass. Cwht1p mutants were partially purified by IMAC and 100 mM imidazole fractions were subjected to Western blot analysis using a monoclonal anti 6×His antibody. Immunoblot analysis confirmed that E580A and D584A had been expressed (Figure 5.14). The band intensity of mutants on SDS-PAGE (Figure 5.13 C) and immunoblot analysis (Figure 5.14, lanes 3 and 4) indicated that mutated proteins were expressed at significantly lower concentrations than the parental Cwht1p. Also, D584A was expressed at a lower concentration than E580A. Further purification of mutated E580A was achieved using the FPLC MonoQ HR5/5 (Figure 5.15 A). SDS-PAGE analysis of fractions obtained from the MonoQ showed that a peak eluted at approximately 0.3 M NaCl (Figure 5.15 A) containing a single band of E580A (Figure 5.15 C, lane3). This was similar to the elution conditions of Cwht1p from the MonoQ (Figure 5.9 A). However, no peak could be detected for D584A at 0.3 M NaCl (Figure 5.15 B).
concentration of E580A and D584A were estimated at 1.2 μg and 0.2 μg per g of wet biomass, respectively, based on the SDS-PAGE analysis and FPLC profile.

5.4.10 E580A and D584A are inactive

The 100 mM imidazole IMAC fractions of E580A and D584A were dialyzed for 8 h in 20 mM phosphate buffer, pH 6.8, and protein concentrated to 0.13±0.05 mg/ml and 0.12±0.05 mg/ml, respectively. Aliquots of 4 μl of the E580A fraction (containing ~ 117 ng of E580A) and D584A fraction (containing ~ 10 ng of D584A) were incubated with 2.5 mM and 10 mM of synthetic trisaccharide for 30 min and 1 h at 37°C. No activity was detected for mutants at either substrate concentration. Furthermore, E580A purified with the MonoQ HR5/5 also did not show any detectable activity with 2.5 and 10 mM synthetic substrate after 1 h incubation at 37°C. No aryl α-glucosidase activity was detected for mutants after IMAC.

5.5 DISCUSSION

Processing α-glucosidase I removes the outermost glucose residues from the newly assembled oligosaccharide soon after en bloc transferring of Glc3Man9GlcNAc2 to the protein (Kornfeld & Kornfeld 1985), and has been suggested to play an important role in protein folding and QC in the ER (Helenius & Aebl 2004).

Yeast α-glucosidase I (Cwh41p) is a type II ER protein with a proposed domain orientation similar to mammalian α-glucosidase I (Jiang et al. 1996; Romero et al. 1997). However, functions of the predicted domains (i.e. N-terminal and transmembrane) are not well understood. Previously it was shown that the soluble form of α-glucosidase I (98 kDa) is released from the membrane-bound enzyme (Cwh41p) during the isolation process by endogenous cleavage between A24-T25, a predicted signal cleavage site (Chapter II).
Moreover, controlled trypsin proteolysis and uncontrolled endogenous hydrolysis of homogeneous and 95% purified soluble α-glucosidase I, respectively, released a mixture of catalytically active polypeptides in which 59 kDa and 37 kDa polypeptides were isolated using CP-DNM based resins (Chapter IV). The 37 kDa polypeptide (F525-F833) was determined to be the smallest proteolytic resistant fragment containing the catalytic domain of the enzyme (Chapter IV). In this study recombinant vectors containing truncated forms of \textit{CWH41} for encoding polypeptides equivalent to these hydrolytic fragments, fused with C-6xHis, were constructed and transformed into \textit{S. cerevisiae}. Immunoblot analyses and α-glucosidase I assay of protein extracts from different recombinant yeasts indicated that Cwht1p (E35-F833) was expressed as a functional polypeptide. However, the 59 kDa fragment (Cwht2p; R320-F833) and the 37 kDa (Cwht3p; M526-F833) containing the catalytic domain, were not detectable by immunoblot analyses. These results suggest that part of the \textit{CWH41} gene encoding E35-M526 of Cwh41p is essential for functional expression. Therefore expression of the non-catalytic region of C-terminal domain of α-glucosidase I likely is important for the catalytic region of enzyme to acquire its active conformation.

Purification of Cwht1p to approximately 92% homogeneity, based on SDS-PAGE analysis, was achieved by a combination of ammonium sulfate precipitation (70%), anion-exchange chromatography (Toyopearl DEAE and FPLC MonoQ HR 5/5), and an affinity chromatography (IMAC). The molecular subunit mass of Cwht1p was determined to be 94 kDa which matches with the theoretical mass of the Cwht1p sequence fused with 6×His residues (93,978 Da). The specific activity of purified Cwht1p was determined to be 3600 U per mg of protein which is comparable to the specific activity of the pure soluble α-glucosidase I (3000 U per mg of protein). This suggests that the region of M1-E33 of
Cwh41p, containing the predicted N-terminal (M1-K10) and the transmembrane domain (T11-I28), does not have any implications for α-glucosidase I activity. As well, protein chimeras fused with the region containing the predicted N-terminal and transmembrane domain of human α-glucosidase I did not affect folding of recombinant proteins (Hardt et al. 2003; Tang et al. 1997). These results together suggest that the predicted N-terminal and transmembrane domain are not important in protein folding. It also indicates that fusion of the enzyme with 6×His residues at the C-terminal does not alter enzyme structure as Cwht1p remained fully functional towards the synthetic trisaccharide.

The soluble α-glucosidase I activity of yeast expressing CWHT1 increased 4-fold per g of biomass compared to yeast overexpressing CWH41. Also, immunoblot analysis did not detect any fraction with Cwht1p in the microsomal protein fraction. As well, although Cwht1p carries the N-glycosylation sites it is found to be not glycosylated. Collectively these findings suggest that Cwht1p was expressed as a soluble fragment that is not associated with the ER membrane, and likely does not translocate into the ER during biosynthesis.

Therefore, the N-terminal and transmembrane domain in yeast α-glucosidase I likely mediate ER localization of α-glucosidase I through an ER signal sequence. In human α-glucosidase I, it was suggested that three N-terminal Arg (R7, R8 and R9) mediate ER localization and therefore play functional role in ER-targeting (Hardt et al. 2003). However, such a region has not been identified within the predicted N-terminus of yeast α-glucosidase I, suggesting other residues likely control translocation of the enzyme into the ER. Furthermore, it has been noted that in type II membrane proteins the hydrophobic region that anchors the protein to the membrane could also be the ER signal sequence suggesting a dual function for that region (Martoglio & Dobberstein 1998). Thus, it is plausible that the hydrophobic region of yeast α-glucosidase I (T11-I28) functions as a signal sequence for translocating protein into the ER.
Unlike the soluble α-glucosidase I, Cwht1p was not glycosylated and this did not have an impact on α-glucosidase I specific activity. Several studies have shown that inhibiting N-glycosylation of some enzymes such as glucose oxidase from *Penicillium amagasakiense* (Witt et al. 1998), ER resident Human 11-beta-hydroxysteroid dehydrogenase type 1 (Blum et al. 2000), and Human 11-beta-hydroxysteroid dehydrogenase type 2 (Kyossev & Reeves 1997), which are natively N-glycosylated, did not affect their enzymatic activity and/or conformation during folding. In addition, it was noticed that treatment of pure Cwht1p with trypsin resulted in the release of a mixture of catalytically active polypeptide(s), suggesting that the catalytic region has remained resistant to hydrolysis in the recombinant fragment. This implies that glycosylation of yeast α-glucosidase I is not crucial for the protein to acquire its active conformation and does not change resistance of the catalytically active region to proteolysis.

It is well accepted that the mechanism of action of glycosyl hydrolases is controlled with a pair of carboxylic acid residues (Koshland 1953). Yeast α-glucosidase I is an inverting enzyme (Palcic et al. 1999) indicating that the mechanism of action is catalyzed by a pair of carboxylic acid residues in which one serves as a general base and other as a general acid (Rye & Withers 2000). Six highly conserved carboxylic acid residues, D601, D602, E613, D617, D670, and E804 of Cwh41p, are situated within the catalytic region (F525-F833) of yeast α-glucosidase I. Furthermore, E613 and D617 (corresponding to E580 and D584 of Cwht1p) are located at the proposed binding motif of mammalian glucosidase I (Figure 5.2), indicating that they may possess a prominent function in enzyme activity. Indeed, site-directed mutagenesis of E580A and D584A (of Cwht1p) resulted in undetectable α-glucosidase I activity with synthetic trisaccharide. It was also shown that neither the mutants nor Cwht1p was able to catalyze p-nitrophenyl α-D-glucopyranoside.
These drawbacks prevented acquisition of important kinetic parameters for E580A and D584A.

However, although inactivation of α-glucosidase I activity could be the result of elimination of carboxylic acid residues acting as general acid and/or general base, it should be noted that the inactivation may also be due to the mutation of other functional carboxylic acid residues. It has been reported that in other inverting glycosidase such as glucoamylase (EC 3.2.1.3, family 15) from *Aspergillus* sp, an α-inverting enzyme, that non-active site carboxylic acid residues such as Asp55 (Sierks et al. 1992; Sierks & Svensson 1993) and Glu180 (Sierks & Svensson 1992) play a functional role by making hydrogen bonds with hydroxyl groups of sugar substrates. It was also reported that site-directed mutagenesis of the non-catalytic Asp101 of β-amylase (EC 3.2.1.2, family 14), a β-inverting enzyme, resulted in undetectable activity (Totsuka et al. 1994). It was noted that Asp101 interacts with the non-reducing terminus of the substrate, adjusting the position of the sugar for catalysis (Ly & Withers 1999).

It was also shown that expression of E580A and D584A was drastically decreased compared to Cwht1p. This problem hindered further structural analysis which usually requires relatively a high quantity of the pure protein. However, it should be noted that Ala is a non-ionizable sterically conserved replacement for the carboxylic acid residues, which has been used frequently in site-directed mutagenesis (Ly & Withers 1999). Presumably, it should have a low impact on structure when it replaces a carboxylic acid residue.

Furthermore, the mutated proteins were eluted in chromatography steps (i.e. IMAC and MonoQ HR5/5) under similar conditions as Cwht1p. This suggests that site-directed mutagenesis likely did not interfere with the conformation of the mutants. However, it should be noted that the possibility of inactivation of enzyme due to changes in structural
conformation associated with site-directed mutagenesis can not be completely excluded. Based on the findings of this study it is not plausible to establish any definite catalytic roles for these carboxylic acid residues but the results of this study clearly indicate that E613 and D617 of yeast \( \alpha \)-glucosidase I are important either for mechanism of action or for establishing the active structural conformation.

5.6 FUTURE STUDIES

Truncated forms of \( CWH41 \) (i.e. \( CWHT2 \) and \( CWHT3 \)), equivalent to the hydrolytic fragments of yeast \( \alpha \)-glucosidase I, were not expressed. Therefore it is important to investigate if these deletions of \( CWH41 \) have any effects on expression and stability of mRNA in the transformed yeast with pCWHT2 and pCWHT3. Furthermore, \( \alpha \)-glucosidase I active conformation is not dependent on glycosylation suggesting the possibility of functional expression of the Cwh41p or Cwhtlp in \( E. coli \). Successful expression of \( \alpha \)-glucosidase I in \( E. coli \) likely can facilitate extraction and purification of the enzyme in a faster manner, and with a higher quantity and purity compared to that from \( S. cerevisiae \).

Inactivation of \( \alpha \)-glucosidase I activity by site-directed mutagenesis of highly conserved E613 and D617 to a non-ionizable Ala indicates the importance of these residues in maintaining enzyme activity. Therefore it is essential to further investigate the role of these two residues by substitution of E613 and D617 with other non-ionizable, sterically conserved residues such as Asn and Gln on \( \alpha \)-glucosidase I activity. The effect of such substitutions on structure and kinetic parameters should be investigated. It will also be important to determine the role of other highly conserved carboxylic acid residues, D601, D602, D670, and E804 in the putative catalytic region of yeast \( \alpha \)-glucosidase I and in other orthologs.
Figure 5.1. Schematic illustration of predicted domains of *S. cerevisiae* α-glucosidase I, Cwh41p (URL: http://ca.expasy.org/cgi-bin/niceprot.pl?P53008). Potential N-glycosylation sites (*) were predicted by NetNGlyc1.0 server (URL: http://www.cbs.dtu.dk/services/NetGlyc/); the highest probability was estimated for Asn at positions 42 and 122, and the lowest for 135, 787, and 805.
Figure 5.2. Alignment of segments of the putative catalytic region of α-glucosidase I that contain conserved carboxylic acid residues. α-Glucosidase I sequences shown are: *Arabidopsis thaliana* (TrEMBL: O64796); *Caenorhabditis elegans* (Swiss-Prot: Q19426); *Schizosaccharomyces pombe* 972h-(Swiss-Prot: O14255); *Drosophila melanogaster* (Swiss-Prot: Q95R68); *Homo sapiens* (Swiss-Prot: Q13724); *Mus musculus* (Swiss-Prot: Q80UM7); *Rattus rattus* (Swiss-Prot: O88941); *Saccharomyces cerevisiae* (Swiss-Prot: P53008). Asterisks indicate the highly conserved carboxylic acid residues; the dotted box shows the proposed binding motif (Romaniouk & Vijay 1997) and arrows are the position of Glu (613) and Asp (617) in *S. cerevisiae* sequence. Dashed lines show regions with no homology with other sequences. Sequence alignment was carried out using T-Coffee version 2.11 (URL: http://igs-server.cnrs-mrs.fr/Tcoffee).
Table 5.1. List of oligonucleotides and primers used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotides/primers</th>
<th>Sequence (5'-3')</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>His-linker</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td>GATCTCATCATCACCATCACCATCCATCAGATgATCTAGAC</td>
<td>5'-phosphorylated, cut form ends with BgII (bold) &amp; XhoI (underlined), stop codon (lower case)</td>
</tr>
<tr>
<td>Antisense</td>
<td>TCGAGTCTAGAtcaATGGGTAGGATGATGATGAGA</td>
<td>5'-phosphorylated, cut form ends with BgII (bold) &amp; XhoI (underlined), stop codon (lower case)</td>
</tr>
<tr>
<td><strong>Amplification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CWHT1</em> (forward)</td>
<td>GAGCCCGAATTCATGGAAGAATATCAAAAGTTCAC</td>
<td>EcoRI (bold), start codon (underlined)</td>
</tr>
<tr>
<td></td>
<td>GAATGAATCTTTTAC</td>
<td></td>
</tr>
<tr>
<td><em>CWHT2</em> (forward)</td>
<td>GAGCCCGAATTCATGGAAGAATATCAAAAGTTCAC</td>
<td>EcoRI (bold), start codon (underlined)</td>
</tr>
<tr>
<td></td>
<td>GATTAC</td>
<td></td>
</tr>
<tr>
<td><em>CWHT3</em> (forward)</td>
<td>AATCCAGAATTCATGACGGAACATCTGAAGGCAAA</td>
<td>EcoRI (bold), start codon (underlined)</td>
</tr>
<tr>
<td></td>
<td>TCC</td>
<td></td>
</tr>
<tr>
<td>Reverse for all</td>
<td>ATACCGAGATCTGAACGCCTCAAGGATGTTGAC</td>
<td>BgII (bold)</td>
</tr>
<tr>
<td><strong>Mutagenic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E580A-sense</td>
<td>CCACCAGATGTAGCagaTTGAACGTAGACGCA</td>
<td>Mutation codon in bold lower case</td>
</tr>
<tr>
<td>E580A-antisense</td>
<td>TGCGTCTACGTCAATgcTGCTACATCTGTTG</td>
<td>Mutation codon in bold lower case</td>
</tr>
<tr>
<td>D584A-sense</td>
<td>TAGCAGAATTGAAACGTaGccGCATTAGCATGCTG</td>
<td>Mutation codon in bold lower case</td>
</tr>
<tr>
<td>D584A-antisense</td>
<td>CACCCATGCTAATGCggctACGTCAATTCTGCTA</td>
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<td><strong>Sequencing</strong></td>
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<tr>
<td></td>
<td>CTCACACACTCTTTTCT</td>
<td>Forward sequencing primer</td>
</tr>
<tr>
<td></td>
<td>TGTAAGAGCAAGAAACGCGG</td>
<td>Forward sequencing primer</td>
</tr>
<tr>
<td></td>
<td>CCAGGGAAACAAGTACGGG</td>
<td>Forward sequencing primer</td>
</tr>
<tr>
<td></td>
<td>TACTCCTGCTCATTAC</td>
<td>Reverse sequencing primer</td>
</tr>
<tr>
<td></td>
<td>GTAAAGGATGGGAAGGAG</td>
<td>Reverse sequencing primer</td>
</tr>
</tbody>
</table>
Figure 5.3. Schematic map of the shuttle vector pHVX2-His. pHVX2-His was constructed by insertion of a double stranded oligonucleotide (His-linker) between BgII and XhoI of pHVX2 (Volschenk et al. 1997) to encode 6×His residues at the C-terminus of the protein.
Figure 5.4. Truncated forms of yeast α-glucosidase I. (A) Amplified DNA fragments (CWHT1-3) of CWH41; (B) Predicted truncated polypeptides (Cwht1-3p) of Cwh41p, shown without the 6×His at the C-terminus.
Figure 5.5. Chemical labeling of glycan with biotin. Glycan was oxidized with sodium periodate (NaIO₄) and probed with biotin hydrazide, adapted from (Zhang et al. 2003).
Figure 5.6. DNA sequencing of truncated forms of CWH41. Shown are the regions of DNA sequence flanking the start codon of CWHT1 (a), CWHT2 (b), CWHT3 (c), and flanking the common His-linker region and stop codon(d). CWHT(1-3) were inserted between EcoRI and BglII sites.
Table 5.2. α-Glucosidase I activity of different yeast transformants.a.

<table>
<thead>
<tr>
<th>Yeast transformant</th>
<th>Activity (U)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHVX2-His</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>pRAN1</td>
<td>300±37</td>
</tr>
<tr>
<td>pCWHT1</td>
<td>1400±180</td>
</tr>
<tr>
<td>pCWHT2</td>
<td>16±4.5</td>
</tr>
<tr>
<td>pCWHT3</td>
<td>8.1±1.4</td>
</tr>
</tbody>
</table>

a Results are the average ± range of two preparations and reported per gram wet weight of yeast biomass (total activity was measured in cell homogenates after removing microsomes by ultracentrifugation)

b 1U of activity = releasing 1 nmol of glucose per min.
Figure 5.7. Single step isolation of proteins from recombinant yeast strains by IMAC. Protein extracts from yeast transformed with control pHVX2-His (I), pCWHT1 (II), pCWHT2 (III), and pCWHT3 (IV) were applied to 1 ml His-Trap column and eluted by step gradient concentration of imidazole, solid lines (A); values above peaks indicate the imidazole concentration (mM); dotted line indicates percentage of buffer B (20 mM phosphate buffer plus 500 mM imidazole and 0.5 M NaCl) in binding buffer. (B) SDS-PAGE (10%) of IMAC fractions. Lane 1, 20; lane 2, 40; lane 3, 60; lane 4, 100; lane 5; 300 mM imidazole fractions. Lane 6 is molecular weight standards. Gels were stained by Coomassie blue.
Figure 5.8. Immunoblot analysis of protein isolates from transformed yeast. (A) SDS-PAGE (10%) of cell homogenates from yeast strains expressing CWHT1 (lane 3), CWHT2 (lane 4), CWHT3 (lane 5), and transformed yeast with pHVX2-His (lane 6). Lanes 1 and 7 were molecular weight standards and lane 2 was protein extracted from microsome of yeast expressing CWHT; gel was stained by Coomassie blue. (B) Western blot analysis was carried out using rabbit anti-yeast α-glucosidase I polyclonal antibody; lane 1, purified Cwhtlp; lane 2-7 as described in figure A. (C) Western blot analysis of proteins were performed using mouse anti-His monoclonal antibody; lane 1, purified Cwhtlp; lane 2-7 as described in figure A. Arrow shows the position of Cwhtlp.
Table 5.3. Purification of Cwht1p from recombinant *S. cerevisiae* expressing *CWHT1*.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Activity (U)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% (NH₄)₂SO₄</td>
<td>1300</td>
<td>9.1</td>
<td>140</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Toyopearl DEAE</td>
<td>830</td>
<td>1.7</td>
<td>490</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>FPLC Mono Q HR5/5</td>
<td>350</td>
<td>0.12</td>
<td>2900</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>FPLC His-Trap</td>
<td>160</td>
<td>0.045</td>
<td>3600</td>
<td>12</td>
<td>7.4</td>
</tr>
</tbody>
</table>

*a* Results are per gram wet weight of yeast cells.

*b* 1 U of activity = 1 nmol of released glucose per min.
Figure 5.9. Purification of Cwhtlp. (A) FPLC MonoQ HR 5/5 chromatogram of partially purified sample from Toyopearl DEAE; α-glucosidase I active fraction (arrow) was eluted at approximately 0.3 M NaCl. Solid line shows protein elution and dashed line indicates percentage of 1 M NaCl in loading buffer. (B) FPLC His-Trap chromatogram of Cwhtlp purified by the MonoQ HR 5/5; α-glucosidase I active fraction (arrow) was eluted by 100 mM imidazole (values above each step gradient shows concentration of imidazole). Solid line shows protein elution and dashed line indicates percentage of buffer B in binding buffer. (C) SDS-PAGE (10%) analysis of Cwhtlp fractions at different stages of purification. Lane 1 and 6, molecular weight standards; lane 2, 70% ammonium sulfate precipitate; lane 3, 0.2 M NaCl α-glucosidase I active fraction from Toyopearl DEAE; lane 4, 0.3 M NaCl α-glucosidase I active fraction from the MonoQ; lane 5, 100 mM imidazole active fraction from His-Trap; lane 7, pure α-glucosidase I from yeast expressing CWH41; lane 1 & 6, molecular weight standards. Gel was Coomassie stained.
Figure 5.10. Cwhtlp is not glycosylated. (A) SDS-PAGE (10%) of the soluble α-glucosidase I (lane 1); soluble α-glucosidase I treated with N-glycosidase F (lane 2); Cwhtlp (lane 3); Cwhtlp treated with N-glycosidase F (lane 4). (B) Western blot analysis of the biotinylated glycoprotein. Same samples as describe in A were treated with NaIO₄ and biotin hydrazide. Samples electrobotted onto PVDF membrane. Immunoblot was carried out using anti-biotin polyclonal antibody conjugated with peroxidase and 4-chloro-1-naphthol as the substrate.
Figure 5.11. Trypsin hydrolysis of pure Cwhtlp at 20°C releases a mixture of α-glucosidase I active polypeptide(s). Pure Cwhtlp was mixed with the modified trypsin (Promega), with the ratio of 8:1 and kept at 20°C for 20 h. Enzyme without trypsin was incubated under the same condition (control). An aliquot of the hydrolyzed enzyme and control were mixed with SDS-PAGE sample buffer, boiled for 5 min, subjected to 12.5 % SDS-PAGE, and Coomassie stained. Cwhtlp only (control, lane 1), hydrolyzed Cwhtlp (lane 2), and molecular weight standards (lane 3). α-Glucosidase I activity was measured for the hydrolyzed enzyme and control after trypsin activity was inhibited with PMSF (100 µg/ml). The values above the figure represent the ratio of total α-glucosidase I activity of the hydrolyzed Cwhtlp compared to control Cwhtlp.
Figure 5.12. Confirmation of E580A and D584A mutations by DNA sequencing. Inserts in plasmids i.e. pCWHT1 and pE580A and pD584A were completely sequenced and the mutations verified. Shown is the region that encodes Asp570-Met593 of Cwht1p (a), E580A (b), and D584A (c). Arrows show the codons of the carboxylic acid residues which were mutated. Asterisks indicate the mutated codons.
Figure 5.13. Isolation of E580A and D584A by IMAC. Protein fractions of three different colonies expressing mutated proteins, E580A (A) and D584A (B), were applied to His-Trap column and proteins were eluted using a step gradient concentration of imidazole. Values above the peaks show the concentration of imidazole (mM). Solid line shows protein elution and dashed line indicates percentage of buffer B in binding buffer. Inserts: colony codes. (C) SDS-PAGE 8-25% (PhastGel) of 100 mM imidazole fractions. Lane (1-3), E580A; lane (4-6), D584A; lane 7, molecular weight standard. The numbers above the image show the codes of the different colonies. Arrows shows the position of the mutated proteins. Gel was stained by Coomassie blue.
Figure 5.14. Western blot analysis of E613A and D617A. 100 mM imidazole IMAC fractions of mutated proteins were run on SDS-PAGE gel (10%) and were electroblotted onto PVDF. Immunoblot was carried out using anti-His monoclonal antibody. Lane 1, Cwht1p; lane 2, pre-stained molecular weight marker; lane 3, E613A; lane 4, D617A. Arrow shows the position of Cwht1p.
Figure 5.15. Purification of mutated proteins by the FPLC MonoQ HR 5/5. 100 mM imidazole IMAC fraction of E580A (A) and D584A (B) after dialysis in 20 mM phosphate buffer (pH 6.8) were applied to the FPLC MonoQ HR5/5. Proteins were eluted by gradient increase in concentration of 1M NaCl in 20 mM phosphate buffer (pH 6.8). Arrow shows the position of E580A, which was eluted at approximately 0.3 M NaCl. (C) SDS-PAGE 8-25% (PhastGel) of protein peaks isolated from the MonoQ. Lane (1-3), proteins from E580A; lane (4 & 5) proteins from D584A; lane 6, molecular weight standards. Letters above the image correspond to the protein peaks observed in (A) and (B). Gel was stained by Coomassie blue.
CHAPTER VI

OVERALL DISCUSSION AND CONCLUSION
6 CHAPTER VI

6.1 OVERALL DISCUSSION

6.1.1 Purification of the soluble form of S. cerevisiae processing α-glucosidase I

A purification method for the soluble α-glucosidase I from yeast overexpressing CWH41 was established based on a combination of ammonium sulfate precipitation, anion-exchange chromatography, lectin (ConA) affinity, and gel-filtration chromatographies. This procedure purified the soluble α-glucosidase I to 95% homogeneity with a low yield (~1%) containing minor impurities including aryl β-glucosidase activity, and the enzyme preparation was rather unstable. The purification method was improved by replacing ConA affinity and size-exclusion chromatography with CP-DNM affinity chromatography. This method resulted in a higher yield (26%) and purity (with no apparent aryl α- or β-glucosidase activity), and the final preparation had a higher stability than the 95% purified enzyme. The ratio of the soluble activity to membrane form in yeast overexpressing CWH41 was determined to be 9:1 which was 9-fold higher than the ratio reported previously for a non-transformed yeast (Kilker, Jr. et al. 1981) demonstrating that overexpression of CWH41 in yeast was concomitant with an increase of the soluble α-glucosidase I activity. Furthermore, the molecular mass of the soluble α-glucosidase I subunit was determined to be 98 kDa. It was also demonstrated that the soluble enzyme is an N-linked glycoprotein.

Growing recombinant yeast in a fermenter and using the improved method for purification of the soluble enzyme resulted in a 40 μg homogeneous preparation per gram of yeast wet biomass. This is the first report that has described purification of the soluble α-glucosidase I to homogeneity.
6.1.2 Soluble α-glucosidase I is a proteolytic product of membrane-bound enzyme

The cleavage between Ala24 and Thr25 of the membrane-bound α-glucosidase I released a soluble activity, and this cleavage can be partially inhibited during the extraction process by inclusion of leupeptin and pepstatin. This is the first direct evidence demonstrating that the soluble form of the enzyme is a proteolytic product of the membrane-bound enzyme encoded by CWH41. It was also noted that the cleavage occurs at the predicted signal peptide cleavage site (Ala24-Thr25) located close to the luminal side of the theoretical transmembrane domain (Thr11-Ile28). Recently, expression of E. coli YgjK protein, a homologue of processing α-glucosidase I, has been reported (Tonozuka et al. 2004). Interestingly, the protein sequencing of expressed YgjK (Swiss-Prot: P42592) indicated that it was a truncated fragment of YgjK produced by endogenous hydrolysis between Ala23-Asn24 of the hydrophobic region. It has been suggested that the M1-A23 may serve as a signal sequence for secretion (Tonozuka et al. 2004). Cleavage at the transmembrane domain which was concomitant with releasing the soluble functional form of the enzyme was also reported for other type II membrane-bound glycosidases residing in the Golgi such as β-1,4-N-acetylgalactosaminytransferase (Jaskiewicz et al. 1996) and sialyltransferase (Lammers & Jamieson 1988).

Endogenous hydrolysis of the integral transmembrane proteins generally takes place by clipping a unique cleavage site which is usually located close to the membrane surface. This releases the bulk of protein often in a fully functional form (Hooper et al. 1997). The cleavage site is specific to the group of proteases called “secretases” and is defined by the topology of the protein substrate (Hooper et al. 1997). Therefore, it is plausible that yapsin proteases, which exhibit secretase like functions in S. cerevisiae (Komano et al. 1999; Krysan et al. 2005), may be involved in clipping the signal cleavage site of α-glucosidase I.
6.1.3 Catalytic region

The soluble α-glucosidase I purified with a lower degree of purity (~95%) was endogenously hydrolyzed to small active fragments that remained fully functional. Also, proteolysis of pure enzyme (100%) using trypsin resulted in the formation of a mixture of catalytically active polypeptide(s) without a loss of activity. Further progressive proteolysis of the pure soluble enzyme with trypsin resulted in the formation of a small resistant polypeptide, a 37 kDa fragment. This fragment, which comprised approximately one third of the C-terminal protein sequence, had higher activity than the intact enzyme (~1.9-fold), and was inhibited by DNM. Increasing activity due to liberation of the catalytic domain by hydrolysis of the intact enzyme was also observed for non-membrane tyrosine phosphatase. Limited proteolysis of human T cell protein tyrosine phosphatase liberated the catalytic domain of the enzyme which had a 20-100 fold increase in activity compared to the full-length enzyme (Hao et al. 1997).

The 37 kDa fragment was isolated from both endogenous and trypsin hydrolysis of the soluble yeast α-glucosidase I. Cleavage sites for the endogenous protease (Arg521) and trypsin (Lys524) were three residues apart suggesting that this region of the Cwh41p may be exposed and susceptible to proteolysis. These cleavage sites are within region G506-N528 of Cwh41p, which has no homology with the sequence of other orthologs from higher eukaryotes. It has been reported that catalytically active polypeptides, were also liberated from mammalian processing α-glucosidase I using limited proteolysis with trypsin (Bause et al. 1989; Shailubhai et al. 1991). However, the exact location of these catalytic regions was not reported prior to this study.

A 39 kDa catalytically active tryptic fragment, which is predicted to be N-glycosylated, was isolated as the smallest resistant polypeptide from rat α-glucosidase I
(Shailubhai et al. 1991). In contrast, the 37 kDa fragment of yeast α-glucosidase I was not glycosylated. The results of this study, and as reported by others for mammalian α-glucosidase I (Bause et al. 1989; Shailubhai et al. 1991), indicate that the catalytic domain of α-glucosidase I in orthologs is resistant to proteolysis.

6.1.4 Potential binding residues

It has been shown that yeast α-glucosidase I activity is sensitive to modification with DEPC, TNM, and NBS, which are selective reagents toward His, Tyr, and Trp, respectively. NEM, a chemical reagent for Cys modification, did not inhibit α-glucosidase I activity. Furthermore, DNM could protect the enzyme against modification with DEPC and TNM but could not provide protection against NBS. These results suggested that His and Tyr may play important functional roles in yeast α-glucosidase I activity. It was reported that α-glucosidase I from mung beans is sensitive to DEPC modification (Zeng & Elbein 1998). In contrast, treatment of α-glucosidase I from pig liver (Zeng & Elbein 1998) and rat liver (Romaniouk & Vijay 1997) resulted in only a slight reduction in activity. Although His residues have not been reported to directly participate in the catalytic mechanism of glycosidases, they have been shown to serve as part of the binding site and to be involved in stabilizing the transition state in several glycosidases (Huber et al. 2001; Sogaard et al. 1993; Watanabe et al. 2001).

Rat mammary α-glucosidase I is also highly sensitive to Tyr modification although neither DNM nor Glc3Man9GlcNAc2 (natural substrate) protected this enzyme against TNM inactivation (Romaniouk & Vijay 1997), suggesting a possible structural role in this ortholog. However, it has been proposed that a unique Tyr/Glu couple likely plays an important role in the mechanism of action of families 33, 34 and 83 of glycoside hydrolases. It has been
suggested the Tyr/Glu couple may serve as a nucleophile in the mechanism of action of these retaining glycosidases (Watts et al. 2003). As of yet, no inverting glycosidase have been shown to use a tyrosine/carboxylic acid residue as a base in their mechanism of actions. Aromatic residues can also be involved in affecting sugar orientation essential for efficient catalysis of the substrate (Matsui et al. 1994). Phe, an aromatic residue, was shown to be important for activity of mammalian α-glucosidase I. It has been reported that the mutation Phe652Leu abolished human α-glucosidase I activity (De Praeter et al. 2000; Volker et al. 2002). This residue corresponds to the conserved aromatic residues in other orthologs (e.g. Tyr668 in S. cerevisiae, Tyr669 in A. thaliana, and Phe588 in C. elegans) suggesting an important role for this residue.

Yeast α-glucosidase I is sensitive to NBS but DNM was not able to protect α-glucosidase I against inactivation. This suggests that Trp may play an important structural role for yeast α-glucosidase I. However, it should be noted that DNM may not protect all important residues. Therefore it is not possible to explicitly determine the role of Trp residue in yeast α-glucosidase I. In contrast, the mammalian enzyme was sensitive to modification of the Trp residue and the activity was protected by DNM against modification (Romaniouk & Vijay 1997).

It was also noted E613LNVDALAW621 of Cwh41p, which corresponds to the proposed binding motif of rat α-glucosidase I (E592 RHLDLRCW600) (Romaniouk & Vijay 1997), does not contain His, Tyr, and Cys residues. These results collectively suggest that the important functional residues (i.e. binding residues) may not be conserved between lower and higher eukaryotes.
6.1.5 Functional domain(s)

A truncated fragment of Cwh41p, lacking the predicted N-terminal region (Met1-Lys10) and transmembrane domain (Thr11-Ile28) and equivalent to the soluble \(\alpha\)-glucosidase I, was functionally expressed. Furthermore, the truncated fragment was not glycosylated and exhibited a comparable specific activity to the soluble \(\alpha\)-glucosidase I. Together, these findings strongly suggest that the Met1-Phe34 region was not required for \(\alpha\)-glucosidase I to acquire its active conformation during biosynthesis.

Since the active fragment (Glu35-Phe833) was not associated with the membrane and was not glycosylated, this suggests that it did not translocate into the ER during biosynthesis. Therefore it is postulated that the region Met1-Phe34 contains a signal sequence that directs Cwh41p into the ER. It has been reported that a protein chimera comprised of the N-terminal and transmembrane domains of human \(\alpha\)-glucosidase I and the luminal domain of the Golgi mannosidase resides in the ER suggesting an important function of those regions for ER localization (Hardt et al. 2003). Since the native yeast \(\alpha\)-glucosidase I in intact yeast cells is associated with the ER membrane (Jiang et al. 1996) it is plausible that the predicted transmembrane domain which exhibits the characteristics of a signal sequence (i.e. consisting of predicted signal cleavage site and comprising the hydrophobic sequence) (Martoglio & Dobberstein 1998) may be important for translocation into the ER.

Other fragments containing the putative catalytic domain of the enzyme (e.g. Arg320-Phe833 and Met526-Phe833) were not expressed. Therefore it was suggested that the Glu33-Phe525 region is likely required for expression of fragments.
6.1.6 Critical carboxylic acid residues (E613 and D617)

Yeast α-glucosidase I is an inverting glycosyl hydrolase (Palcic et al. 1999). Therefore, the catalytic mechanism of the enzyme requires a pair of carboxylic acid residues that serve as a general base and a general acid (Rye & Withers 2000). It has been found that six highly conserved carboxylic acid residues (D601, D602, E613, D617, D670, and E804) are situated within the putative catalytic region of yeast α-glucosidase I. E613 and D617 are situated at the corresponding proposed binding motif of mammalian α-glucosidase I (Romaniouk & Vijay 1997), which suggests that E613 and D617 could be prominent residues for enzyme activity. Indeed, it was observed that substitution of E580 and D584 of Cwht1p, corresponding to E613 and D617 of Cwh41p, with Ala, resulted in inactivation of enzyme when assayed with synthetic trisaccharide. Also, it was observed that mutations of these two highly conserved carboxylic acid residues drastically reduced the expression level of enzyme variants.

A homology search based on the predicted tertiary structure of yeast α-glucosidase I using “Phyre”, a web-based software available online at http://www.sbg.bio.ic.ac.uk/~phyre/, revealed that yeast α-glucosidase I has some regions with significant structural homology with bacterial glucoamylase and chitobiose2 phosphorylase (from Vibrio proteolyticus). However, it was noted that the predicted structure for the D601-D617 region did not share any similarity with any of these proteins, suggesting that this region possesses unique folding characteristics.

Therefore, with the current results it is not possible to assign any definite catalytic or structural roles for E613 and D617. Nevertheless, it is apparent that these conserved residues are playing an important role in maintaining α-glucosidase I activity.
6.2 OVERALL CONCLUSION

Processing α-glucosidase I (EC 3.2.1.106) is the first glycosyl hydrolase that cleaves the outermost glucose residue from the newly assembled Glc₃Man₃GlcNAc₂ and belongs to the family 63 of glycosyl hydrolases. It has been suggested that this enzyme plays an integral role in the N-glycosylation pathway and quality control in virtually all eukaryotes (Herscovics 1999a; Herscovics 1999b). Function of this enzyme has been found to be crucial for cell vitality and normal development in higher eukaryotes such as Arabidopsis and humans (Boisson et al. 2001; De Praeter et al. 2000).

However, instability of the enzyme, difficulties in purification, low quantity of pure native enzyme, and substrate limitations have hampered studies. Although α-glucosidase I genes have recently been identified in humans, Arabidopsis, and S. cerevisiae, it has been noted that expression of the recombinant enzyme from higher eukaryotes did not show promising results. Expression of human α-glucosidase I, for instance, was concomitant with a 4-fold increase in activity (Kalz-Fuller et al. 1995). In contrast, expressing CWH41 in S. cerevisiae resulted in a 28-fold increase in activity (Dhanawansa et al. 2002). Furthermore, yeast α-glucosidase I shares an overall high identity (34-49%) with orthologs from higher eukaryotes at some regions of the C-terminus domain (Romero et al. 1997). Also, it has been noted that α-glucosidase I from lower and higher eukaryotes exhibit comparable physiological roles, biochemical properties, and overall structural similarities. Hence, yeast α-glucosidase I can be a good model for further structural and mechanistic investigation of family 63.

It was reported that the α-(1,2) glucosidase activity in yeast could be isolated as a soluble form as well as a membrane-associated form (Kilker, Jr. et al. 1981). Results of this study demonstrated that the yeast soluble α-glucosidase I is liberated from the membrane-
bound enzyme and indeed is not expressed by a separate gene. Furthermore, it was shown that clipping occurs at the theoretical signal peptide cleavage site of the predicted transmembrane region. It was noted that the predicted signal peptide cleavage site is situated close to the lumen of the ER but it is not exposed as it was shown that the soluble form can not be detected in undisrupted yeast cells (Jiang et al. 1996). These findings, taken together, suggest that the putative transmembrane region may act as a signal sequence for ER-targeting. Indeed, expression of the truncated form of the enzyme without an N-terminal region and a transmembrane domain as a non-glycosylated soluble active fragment indicates that an ER-targeting signal resides within these regions. Expression of a catalytically active and non-glycosylated fragment of yeast α-glucosidase I, which exhibited a comparable specific activity to its glycosylated isoform, indicates that glycosylation is not essential for the enzyme to acquire its active conformation. Based on these findings, the possibility of functional expression of the yeast α-glucosidase I under different promoters using an E. coli expression system should be investigated. Expression of the soluble form of yeast α-glucosidase I in E. coli may provide milligram(s) of enzyme per litre of culture (compared to 400 μg of pure enzyme per litre of culture of yeast), that can be used for crystallographic studies.

The results of this study demonstrated that hydrolysis of the yeast soluble α-glucosidase I (98 kDa) resulted in the release of a 37 kDa catalytically active fragment. It was noted that formation of the 37 kDa active fragment was concomitant with a considerable increase in total activity, up to 1.9-fold. Therefore it is important to elucidate if this phenomenon is related to conformational changes in that region that occur upon hydrolysis. It was also shown that a 39 kDa catalytically active fragment can be isolated from mammalian enzyme (85 kDa) (Shailubhai et al. 1991). This suggests that the catalytic region
of enzymes from lower and higher eukaryotes is resistant to hydrolysis and likely possesses some conserved structural features. Furthermore, it was shown that the smaller proteolytic fragments (i.e. 59 kDa and 37 kDa) were not expressed in \textit{S. cerevisiae}. This draws attention to the importance of the non-catalytic region of the protein and its corresponding cDNA in the functional expression of these fragments. Therefore, it is recommended to determine if the truncation of DNA had an impact on expression and stability of mRNA in a transformed yeast. However, current findings demonstrate that when the C-terminal domain of \(\alpha\)-glucosidase I is expressed in almost full sequence, the catalytic region of enzyme can acquire an active conformation that is resistant to hydrolysis and can be released by proteolysis as a 37 kDa catalytically active fragment.

His and Tyr likely play important roles in yeast \(\alpha\)-glucosidase I. It was also shown that the Cys residue is not important for yeast activity. These results indicate that functional residues are more conserved between yeast and plant enzymes than the mammalian enzyme. Alignment of \(\alpha\)-glucosidase I sequences from yeast and plant sources revealed a single conserved His residue in the catalytic region at position 686 of Cwh41p. This residue was aligned with Ala from mammalian. Therefore, it is suggested that the function of His686 in yeast \(\alpha\)-glucosidase I be further investigated using site-directed mutagenesis.

Mutation of two highly conserved carboxylic acid residues (E613A and D617A) within the region corresponding to the proposed binding motif of mammalian enzyme suggested an important role for these residues in enzyme functionality. To have a better understanding about the function of these residues it is essential to replace these residues with other non-ionizable sterically conserved residues, such as Gln and Asn, and evaluate the effect of these mutations on kinetic parameters as well as structural conformation. Furthermore, the function of four other highly conserved carboxylic acid residues residing in
the putative catalytic region (i.e. D601, D602, D670, and E804) should be investigated using site-directed mutagenesis.

Overall similarity in the functional-structural characteristics of α-glucosidase I orthologs, ability for large scale production of the yeast enzyme, and stability of the pure recombinant yeast enzyme compared to α-glucosidase I from higher eukaryotes indicates that the yeast processing α-glucosidase I is a promising model for investigating the mechanism of family 63 of glycosyl hydrolases. In addition, investigation of the structure of this enzyme may allow us to obtain a better understanding about family 37 glycosyl hydrolases (trehalase, EC 3.2.1.28), which along with family 63, belongs to clan GH-G.

Current inhibitors of α-glucosidase I were first identified from natural sources such as bacteria (e.g. DNM) and plants (e.g. castanospermine). Therefore it seems, the yeast enzyme can be used for rapid screening of other natural extracts with the aim of identifying more potent inhibitors. It also can be used for designing more specific inhibitors that are very important tools for elucidation of the mechanism of action in glycosidases.

In the glycomics era studies have been focused on investigating the role of carbohydrate in different crucial biological processes (i.e. signal transduction, cell-cell interactions, viral entry, and bacteria-host interactions), and it is essential to understand the structure of the oligosaccharides. However, structural diversity and complexity of carbohydrates and glyco-conjugates have hindered glycomics studies. Glycosidases are among the most prominent tools used for glycomics studies; the specificity of these enzymes allows researchers to acquire valuable information about carbohydrate structures (Ratner et al. 2004). Therefore, it seems yeast α-glucosidase I can be used as an important tool for glycomics studies that have targeted the early steps of the N-glycosylation and also as a
model to elucidate important mechanistic information about the family 63 glycoside hydrolases.
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APPENDICES
APPENDIX 1

Yeast transformation procedure

Yeast was grown overnight in 5 ml YPD in an incubator shaker (30°C and 350 rpm) and 2.5 ml was used for inoculation into 250 ml of fresh YPD, in a 1 litre flask. The culture was grown at the same conditions until it reached OD_600 of 0.8. Cells were harvested at 7,000×g for 10 min in a sterile centrifuge tubes. The cells pellet was resuspended in 10 ml sterile TL buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM lithium acetate; autoclave-sterilized), and the suspension was transferred to a 35 ml sterile centrifuge tube and centrifuged at 7,000 × g for 10 min. Cells were then resuspended in 10 ml fresh TL buffer and incubated at 30°C for 1h with shaking at 100 rpm. The suspension was centrifuged at 5,000 × g for 5 min and the cells resuspended in 2 ml fresh TL buffer. Aliquots of 100 µl were transferred into sterile microfuge tube for each transformation reaction. Plasmid (1 µg) and 5 µl of 10 mg/ml sheared salmon sperm carrier DNA were added to each tube, mixed gently and incubated at 30°C for 30 min. TLP (400 µl; 10 mM Tris-HCl pH 7.5, EDTA 1 mM, 100 mM lithium acetate, 44% polyethylene glycol 4000; autoclave-sterilized) was added, mixed gently, and incubated at 30°C for 60 min. Cells were heat shocked by incubating tubes in a 42°C water bath for 5 min. Tubes were microfuged for 10 second at the maximum speed and the supernatant removed. Pellets were gently resuspended in 300 µl TL buffer. A 100 µl aliquot was transferred onto a plate containing minimal medium with L-histidine (50 mg/L) and agar (1.5% w/v). Plates were incubated for 48-72 h at 30°C until colonies reached 1 to 2 mm in diameter and then were subcultured onto selective plates. This method was adapted from Gietz and co-workers (Gietz et al. 1992; Schiestl & Gietz 1989).
APPENDIX 2

Anti-yeast α-glucosidase I polyclonal antibody

Polyclonal antibody against the soluble form of yeast α-glucosidase I was raised for immunoblot detection of the enzyme and its fragments. α-Glucosidase I was purified according to the method described in Chapter III. Purified enzyme was further polished by FPLC Superdex 200 gel-filtration column, dialyzed in deionized distilled water, freeze dried, and aliquots of 100 µg were kept at -80°C. A 100 µg sample of purified enzyme was dissolved in 1 ml of water and mixed with 1 ml of Freund’s complete adjuvant. The emulsion was then injected into multiple sites intradermally on the back of a female New Zealand Rabbit. One booster injection of 100 µg of enzyme in Freund’s incomplete adjuvant was conducted after 2 weeks. Blood was collected one week after the booster injection and serum was separated and kept at -80°C.

Specificity of the antibody for yeast α-glucosidase I was tested by Western blotting. Partially purified α-glucosidase I from Toyopearl DEAE was subjected to SDS-PAGE under reducing conditions and electro-blotted on PVDF membrane. Different dilutions of serum containing the IgG were used to detect α-glucosidase I. Goat anti-rabbit polyclonal antibody conjugated with peroxidase was used as secondary antibody to detect rabbit polyclonal antibody. 4-Chloro-1-naphthol was used as the peroxidase substrate.
Appendix 2.1. Immunoblot analysis with rabbit anti-yeast α-glucosidase I polyclonal antibody. An equal amount of protein (~ 5 μg) of glucosidase I active fraction from Toyopearl DEAE was electro-blotted on PVDF membrane and stained with Ponceau S solution (Sigma) (A). PVDF membrane was destained with 0.1 M NaOH and immunoblotting was followed by standard Western blot protocol using different concentrations of rabbit serum. The reaction was completed using goat anti-rabbit antibody conjugated with peroxidase (B). Arrows show the positions of the soluble α-glucosidase I. Ratios shown below the image were dilution factors of rabbit serum.