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Truncations and functional carboxylic acid residues of yeast processing α-glucosidase I

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Abstract Yeast α-glucosidase I (Cwh41p) encoded by *CWH41* is an endoplasmic reticulum (ER) membrane-bound glycoprotein (833 residues), which plays an important role in the early steps of the *N*-glycosylation pathway. In this study functional expression of three truncated fragments of Cwh41p, all containing the catalytic region, was investigated. Cwht1p (E35-F833), with deletion of the N-terminus and transmembrane domain, was expressed as a catalytically active fragment while R320-F833(Cwht2p) and M526-F833 (Cwht3p) were not detected. Significantly higher glucosidase I activity was found in a soluble extract from yeast over-expressing *CWHT1* (1,400 U/g biomass) than yeast over-expressing *CWH41* (300 U/g biomass). Cwht1p was purified as a soluble 94 kDa non-glycosylated protein with a specific activity (3,600 U/mg protein) comparable to that of the soluble α-glucosidase I (3000 U/mg protein). These findings indicate that the active conformation of the enzyme is not dependent on protein glycosylation and suggest that the M1-I28 region of Cwh41p carries an ER-targeting signal sequence. In addition, two highly conserved carboxylic acid residues, E580 and D584 of Cwht1p (corresponding to E613 and D617 of Cwh41p), located within the catalytic domain of yeast enzyme were subjected to mutation. Substitution of each residue with Ala resulted in low expression and undetectable glucosidase I activity. These findings indicate that E613 and D617 play a crucial role in maintaining α-glucosidase I activity.

Keywords *CWH41* · Functional domains · *Saccharomyces cerevisiae* · Site-directed mutagenesis · Processing α-glucosidase I

Abbreviations

CWHT	truncated form of *CWH41*
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FPLC	fast protein liquid chromatography
IMAC	immobilized metal affinity chromatography
OD	optical density
PMSF	phenylmethanesulfonyl fluoride
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Mannosyl-oligosaccharide glucosidase or processing α-glucosidase I (EC. 3.2.1.106) is the exclusive member of family 63 glycoside hydrolases [1]. Despite the key role of α-glucosidase I in the *N*-glycosylation pathway it has remained one of the least characterized glycoside hydrolases in the ER due to the unique substrate specificity, and difficulties with purification, and over-expression in higher eukaryotes [2, 3]. Furthermore, family 63 does not show any significant structural similarity with other known

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families of glycosyl hydrolases. Therefore, limited mechanistic-structural information about family 63 can be inferred from other known families of glycosyl hydrolases.

Yeast α-glucosidase I (Cwh41p), is a type II integral membrane N-glycoprotein (833 residues) encoded by *CWH41* [4, 5]. Cwh41p (Swiss-Prot: P53008) is predicted to have a short cytosolic N-terminus domain (M1-K10), a hydrophobic transmembrane domain (T11-I28), and a large C-terminus domain (S29-F833) extended toward the lumen of the ER. Previously, we demonstrated that endogenous cleavage at the predicted signal sequence of the transmembrane region of Cwh41p liberated the soluble form of α-glucosidase I activity [6]. Furthermore, catalytically active fragments, 59 and 37 kDa, were isolated from the soluble α-glucosidase I during endogenous hydrolysis or trypsin proteolysis of the enzyme [7, 8]. The 37 kDa polypeptide (F525-F833) appeared to be the smallest non-glycosylated catalytically active fragment [8]. Therefore, in this study functional expression of truncated forms of the *CWH41* gene—*CWHT1, CWHT2, and CWHT3*—encoding, respectively, polypeptides E35-F833, R320-F833, and M526-F833 fused with C-6xHis tag, were investigated. These correspond to the soluble enzyme, and the 59 and 37 kDa active fragments.

It was reported that α-glucosidase I is an inverting glycosyl hydrolase [9]. This process is catalyzed by a pair of carboxylic acid residues [10], where one carboxylic acid serves as a general acid and the other as a general base [11]. 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 of the yeast enzyme. Furthermore, E613 and D617 are located within the proposed substrate binding motif of rat α-glucosidase I (E592 RHLDLRCW600) [12], which corresponds to E613-W621 of Cwh41p. Therefore, the importance of these residues in yeast α-glucosidase I were investigated by substitution with Ala. This study provides better insight into the functional relationship of the different domains of yeast α-glucosidase I. As well, it shows the significance of two highly conserved carboxylic acid residues (E613 and D617) for maintaining catalytic and/or structural integrity of the enzyme.

Materials and methods

Materials

All restriction enzymes, rapid DNA ligation kit, bacterial transformation kit (TransformAid), and DNA ladder (Mass Ruler) were obtained from Fermentas. Polymerase chain reaction (PCR) purification, DNA gel extraction, and plasmid purification kits, His-linker oligonucleotides, PCR, and mutagenic primers were purchased from Qiagen. Low melting point agarose (Ultrapure) and salmon sperm DNA were products of Gibco-BRL. Oligonucleotides for DNA sequencing were synthesized at the Nucleic Acids and Protein Service, University of British Columbia. dNTPs were purchased from GE Healthcare. MilliQ ultra pure water was used for all of the steps involved in DNA preparation and manipulation. Mouse anti 6xHis-tag monoclonal antibody was from Novagen, peroxidase-conjugated goat anti-mouse polyclonal antibody was obtained from BD Biosciences, peroxidase-conjugated goat anti-rabbit polyclonal antibody and 4-chloro-1-naphthol were products of Sigma.

Microbial strains and media

E. coli DH5-α (λ^- *end*A1, *hsd*R17, supE44, *thi*-1 1^-, *rec*A1, *gyr*A96, *rel*A1, •*lac*U169 [φ80d *lac* ZDM15]) and *Saccharomyces cerevisiae* AH22 (*MAT a, Leu2-3,leu2-112, his4-519, canI, [cir+]*) were used. Transformed and non-transformed *E. coli* strains were grown in Luria-Bertani (LB) broth with or without ampicillin (100 μg/ml), respectively. *S. cerevisiae* AH22 was grown in YPD (1% yeast extract (Difco)+ 2% Bacto-peptone (Difco)+ 2% glucose). Transformed yeast strains were grown in minimal medium plus 50 mg/l L-histidine [6] until a turbidity (OD_{600}) of 4 was reached with the average yield of 6.9± 0.6 g of wet biomass per litre of culture.

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 [13].

Plasmids

pRAN1, a pHVX2 vector carrying *CWH41* [6] was used as the template for construction of truncated fragments by PCR. pHVX2 is a shuttle vector containing phosphoglycerate kinase gene (*PGK1*), a constitutive promoter [14].

Construction of pHVX2-His

Two complementary 5′-phosphorylated strands of oligonucleotide in the cut form with *Bgl*II (5′end) and *Xho*I (3′end), encoding six His residues (His-linker), were designed (Table 1). For annealing, 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 $MgCl_2$, 10 mM DTT) was added. The mixture was heated to 95°C for 5 min in a thermal cycler

Table 1 List of oligonucleotides and primers used in this study

Oligonucleotides/ primers	Sequence (5′-3′)	Notes
His-linker		
Sense	**GATCT**CATCATCACCATCACCATtgaTCTAGAC	5′-phosphorylated, cut form ends with *Bgl*II (bold) & *Xho*I (underlined), stop codon (lower case)
Antisense	TCGAGTCTAGAtcaATGGTGATGGTGATGATG**A**	5′-phosphorylated, cut form ends with *Bgl*II (bold) & *Xho*I (underlined), stop codon (lower case)
Amplification		
CWHT1 (forward)	GAGCCC**GAATTC**ATGGAAGAATATCAAAAGTTCACGAATGAATCTTTAC	*EcoR*I (bold), start codon (underlined)
CWHT2 (forward)	GAGCCC**GAATTC**ATGAGGGAACAAGTTACGGAGTTGATTAC	*EcoR*I (bold), start codon (underlined)
CWHT3 (forward)	AATCCA**GAATTC**ATGACGAACAATCTAGAAGCCAATCC	*EcoR*I (bold), start codon (underlined)
Reverse for all	ATACCG**AGATCT**GAAGCGTCCAAGGATGTTGAC	*Bgl*II (bold)
Mutagenic		
E580A-sense	CCACCAGATGTAGCA**gca**TTGAACGTAGACGCA	Mutation codon in bold lower case
E580A-antisense	TGCGTCTACGTTCAA**tgc**TGCTACATCTGGTGG	Mutation codon in bold lower case
D584A-sense	TAGCAGAATTGAACGTA**gcc**GCATTAGCATGGGTG	Mutation codon in bold lower case
D584A-antisense	CACCCATGCTAATGC**ggc**TACGTTCAATTCTGCTA	Mutation codon in bold lower case
Sequencing		
	CTCACACACTCTTTTCT	Forward sequencing primer
	TGTAAAGAGCAAGAACGGGG	Forward sequencing primer
	CCAGGGAACAAGTTACGGAG	Forward sequencing primer
	TACTCCTTGCCTCATTACCC	Reverse sequencing primer
	GTAAAGGATGGGGAAAGAG	Reverse sequencing primer

(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 *Bgl* II and *Xho*I, purified with the gel extraction kit, and 50 ng (~16–18 μl) mixed with 1 μl of diluted annealing mixture. 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 *EcoR*I and *Bgl*II cloning sites, was used for expression. 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.

Construction and subcloning of truncated forms of *CWH41*

Truncated forms of *CWH41*(2,502 bp), *CWHT1* (2,400 bp), *CWHT2* (1,542 bp), and *CWHT3* (924 bp), were amplified by PCR. Upstream primers were synthesized with an *EcoR*I restriction site at the 5′ end and down stream primers with a *Bgl*II site at the 5′ end (Table 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 10xPCR 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 the manufacturer's protocol. The size of PCR products were verified by agarose gel electrophoresis stained with ethidium bromide. Amplified DNAs were purified using the PCR purification kit, cut with *EcoR*I and *Bgl*II, and purified by a gel extraction kit. Ligation of *CWHT(1–3)* with the cut and purified 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.

Extraction, isolation, and purification of Cwht(1–3)p

The soluble fraction of protein was extracted from the transformed yeast cells as described in a previous report [6]. α-Glucosidase I activity was measured in the microsomal free supernatant. Soluble proteins were precipitated with 70% saturated ammonium sulfate and protein precipitant was solubilized in 20 mM sodium phosphate, pH 6.8. 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 1 ml His-Trap HP column (GE Healthcare) equilibrated 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. Higher level of purity was obtained using a combination of anion-exchange chromatographies [7] and IMAC.

Anti-yeast α-glucosidase I polyclonal antibody

Purified soluble α-glucosidase I (100% homogeneity by Coomassie blue stained SDS-PAGE gel) was 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 (Sigma). The emulsion was then injected into multiple sites intradermally on the back of a female New Zealand rabbit (Animal Care Centre, University of British Columbia). One booster injection of 100 μg of enzyme in Freund's incomplete adjuvant (Sigma) was made after 2 weeks. Blood was collected 1 week after the booster injection and serum was separated and kept at −80°C. Specificity of the antibody was tested by Western blotting of partially purified yeast α-glucosidase I (results not shown).

Immunoblot analysis

Proteins from different transformed yeasts were transferred onto PVDF membrane 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 and/or mouse anti 6xHis-tag monoclonal antibody according to the manufacturer's procedure (Novagen) with detection using peroxidase conjugated goat anti-mouse polyclonal antibody or peroxidase conjugated goat anti-rabbit polyclonal antibody, and 4-chloro-1-naphthol as the substrate.

Detection of glycoproteins

Aliquots of proteins were deglycosylated with N-glycosidase F as described previously [7]. 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 ($NaIO_4$) at a final concentration of 10 mM. After reacting for 30 min at 4°C, the solution was dialyzed as noted above, mixed with biotin hydrazide at a final concentration of 2 mg/ml, and incubated for 2 h at room temperature. Samples were run on SDS-PAGE, electroblotted onto PVDF membrane and reacted with peroxidase conjugated anti-biotin goat IgG (Rockland Immunochemicals, Inc.), according to the manufacturer's procedure.

Site-directed mutagenesis of carboxylic acid residues

The PCR reaction was comprised of 50 ng of pure ~9.9 kb pCWHT1 (parental plasmid), 125 ng of mutagenic primers (Table 1), 0.25 mM (final concentration) dNTPs, and 5 μl of 10xPCR buffer; the final volume was adjusted to 50 μl with water before adding 2.5 U of *pfuUltra* high-fidelity DNA polymerase (Stratagene). PCR thermal cycling was initiated with a denaturing cycle (1 min at 95°C), followed by 17 cycles (50 sec at 95°C, 55 sec at 56°C, and 10 min at 68°C). The PCR products were treated with 15 U of *Dpn*I and incubated for 3–6 h at 37°C. Mutated plasmids were purified and transformed into *E. coli* DH5-α. pE580A and pD584A were purified from transformed *E. coli*, mutations were verified by DNA sequencing, and transformed into *S. cerevisiae* AH22.

Extraction, isolation, and detection of E580A and D584A

Three colonies from yeasts transformed with pE580A and pD584A were selected randomly. Cultures grown for 25 h at 28°C with shaking at 300 rpm reached an average turbidity (OD_{600}) of 3.3±0.5 and 3.5±0.6 for pE580A and pD584A yeast strains, respectively. Extraction of proteins was carried out using the method described previously [6] and isolated by IMAC as described for Cwht1p. Expression of mutated proteins was detected by immunoblotting using an anti 6xHis monoclonal antibody. Colonies with potentially higher expression level, according to the SDS-PAGE profile were grown in 1 l of media and protein was extracted from approximately 7 g of wet biomass. The protein after IMAC was further purified by chromatography on the FPLC MonoQ HR 5/5.

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-$(CH_2)_8COOCH_3$, and α-nitrophenyl α-D-glucopyranoside, respectively, as described elsewhere [7].

Results

Cwht(1–3)p isolation and detection

Transformed yeast strains with pHVX2-His (a plasmid without insert) and pCWHT(1–3), (pHVX2-His carrying truncated forms of *CWH41*) had a lower growth rate than the yeast transformed with pRAN1 (pHVX2 carrying *CWH41*) [6].

The soluble α-glucosidase I activity was determined in microsome-free cell extracts of yeast strains transformed with pCWHT1-3. Total activity of the transformed yeast strain expressing *CWHT1* was 1,400 U per g of wet biomass, which is approximately 4-fold higher than the yeast expressing *CWH41* (300 U/g wet biomass). Other transformed yeast strains possessed negligible α-glucosidase I activity—16 and 8 U/g wet biomass for *CWHT2* and *CWHT3*, respectively—indicating that only Cwht1p was catalytically active. Therefore, Cwht2p and Cwht3p either were not expressed or were not functional.

Fractions eluted from the His-Trap column from yeast transformed with either the plasmid carrying *CWHT2* or *CWHT3* were identical to those from the control plasmid, pHVX2-His, indicating that these truncated glucosidase I forms were not expressed (Fig. 1). Only the 100 mM imidazole fraction of yeast expressing *Cwht1p* had a protein band corresponding to the expected molecular weight of the gene product, and this was the only fraction with α-glucosidase I activity. Western blot analyses of cell homogenates with anti-yeast α-glucosidase I antibody and anti-His antibody only detected Cwht1p in the soluble fraction and not in microsomal proteins (Fig. 2).

Purification of Cwht1p

Immobilized metal affinity chromatography (IMAC) alone was not able to purify Cwht1p (Fig. 1 (II), lane 4). Purification of Cwht1p to approximately 90% 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 IMAC. Table 2 shows the purification results. Purification yield was 12%, or approximately 45 μg of Cwht1p per g of wet biomass. The molecular mass of the purified Cwht1p subunit appeared to

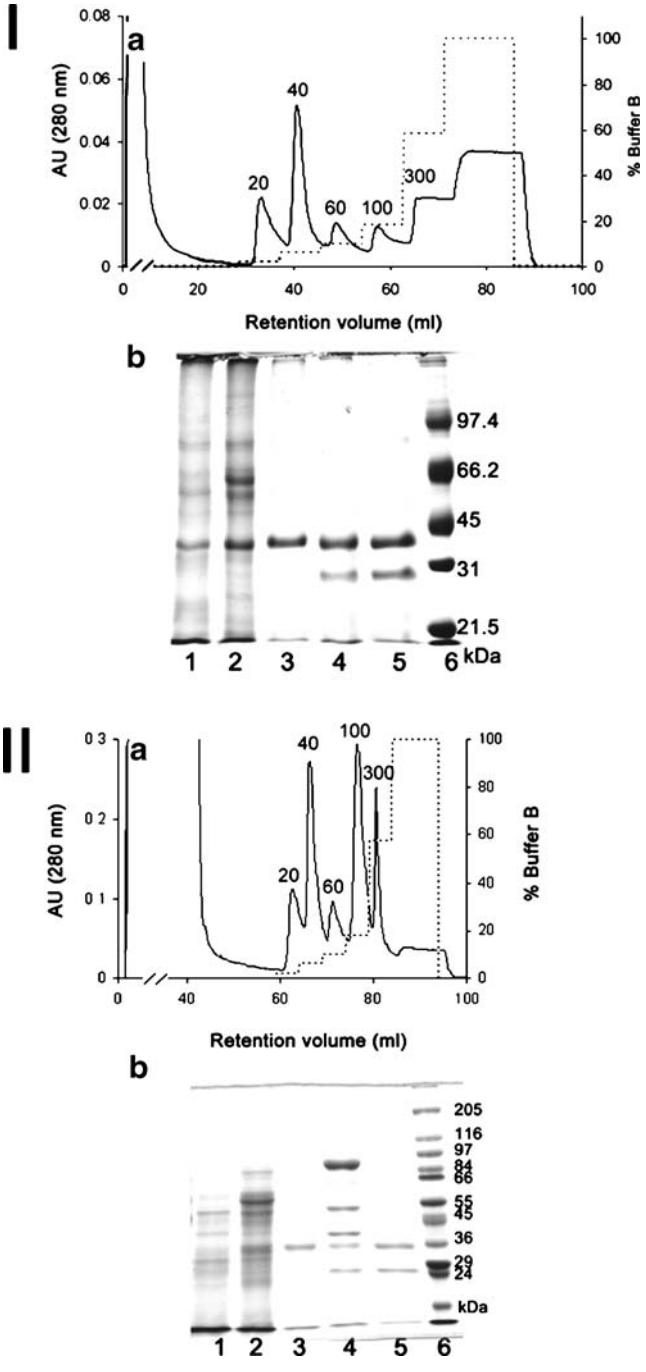

Fig. 1 Single-step isolation of proteins from transformed yeasts by IMAC. Soluble extracts from yeast transformed with pHVX2-His (I), and pCWHT1 (II) were applied to His-Trap column and eluted by step gradient concentration of imidazole, *solid lines* **a**; values above peaks indicate the imidazole concentration (mM); *dotted line* indicates percentage of buffer B (20 mM phosphate buffer plus 500 mM imidazole and 0.5 M NaCl). **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. Panel I was similar to the profiles for Cwht(2 and 3)p

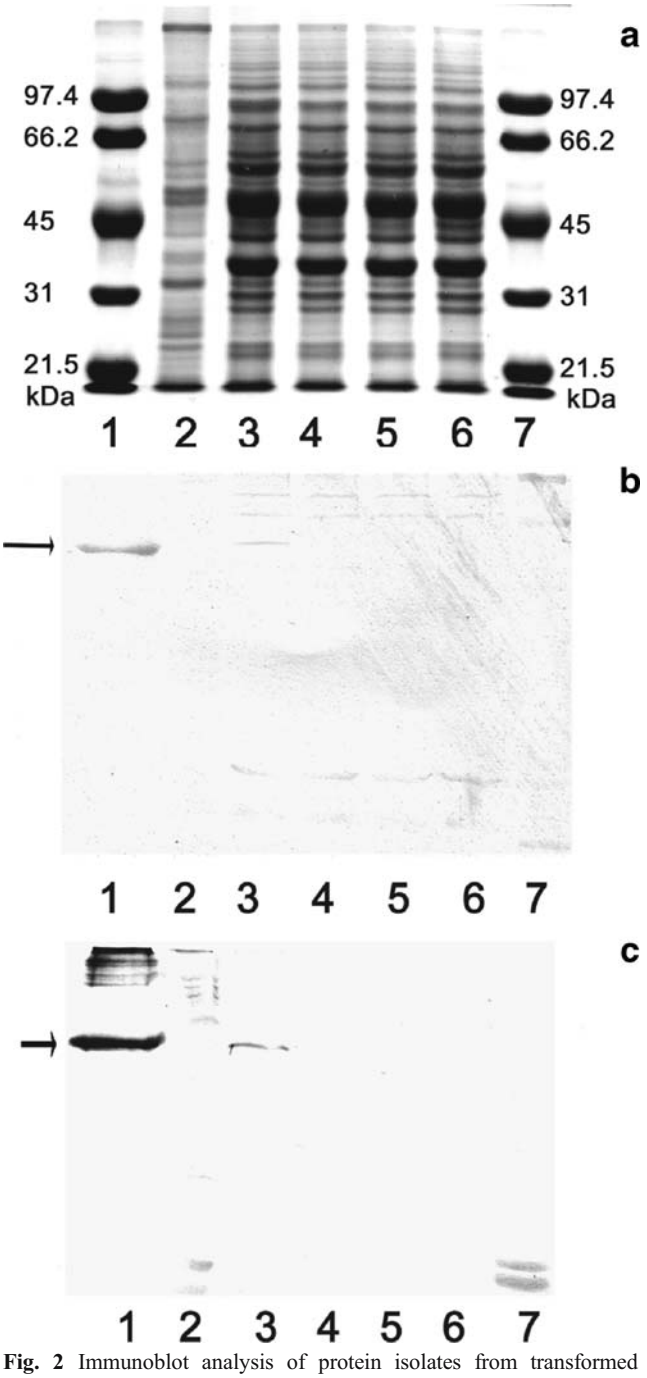

Fig. 2 Immunoblot analysis of protein isolates from transformed yeasts. **a** SDS-PAGE (10%) of cell homogenates from yeast strains expressing *CWHT1* (lane 3), *CWHT2* (lane 4), *CWHT3* (lane 5), and control yeast with pHVX2-His (lane 6). Lanes 1 and 7 were molecular weight standards and lane 2 was protein extracted from microsome of yeast expressing *CWHT1*; 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 Cwht1p; lane 2–7 as described in **a**. **c** Western blot analysis of proteins were performed using mouse anti-His monoclonal antibody; lane 1, purified Cwht1p; lane 2–7 as described in **a**. *Arrow* shows the position of Cwht1p

be 94 kDa on SDS-PAGE (Fig. 3) which matches with the theoretical mass of the Cwht1p sequence fused with 6xHis residues of 93,978 Da.

Cwht1p is not glycosylated

It was observed that the molecular mass of the soluble α-glucosidase I treated with N-glycosidase F was reduced while no change was detected for Cwht1p (Fig. 4a). Confirmation that Cwht1p was not glycosylated was obtained by specific chemical biotinylation of carbohydrate followed by immunodetection (Fig. 4b).

Releasing catalytically active polypeptides by hydrolysis of Cwht1p

Overnight hydrolysis of pure Cwht1p with trypsin released a mixture of catalytically active polypeptides (Fig. 5). Total activity of this Cwht1p hydrolysate was determined to be 1.4-fold higher than Cwht1p. A similar increase of activity was also reported for endogenous and trypsin hydrolysis of the soluble α-glucosidase I [7, 8]. The polypeptide bands appeared to have molecular masses of 54, 38, 29, and 24 kDa, with the 38 kDa band having the highest intensity.

Isolation, detection, and purification of Cwht1p mutants (E580A and D584A)

Enzyme mutants were isolated using IMAC with an imidazole step-gradient elution as described for Cwht1p. The 100 mM imidazole fraction had multiple protein bands, including the protein of interest. Immunoblot analysis of 100 mM imidazole fractions with a monoclonal anti 6xHis antibody confirmed that E580A and D584A were expressed (Fig. 6). The band intensity on SDS-PAGE and immunoblot analysis (Fig. 6, 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 E580A was achieved using the FPLC MonoQ HR5/5. SDS-PAGE analysis of fractions obtained from the MonoQ showed that a peak eluted at approximately 0.3 M NaCl containing a single band of E580A. This was similar to the elution conditions of Cwht1p from the MonoQ. However, no peak could be detected for D584A at 0.3 M NaCl. The concentration of E580A and D584A were estimated at 1.2 μg and 0.2 μg/g wet biomass, respectively, based on the SDS-PAGE analysis and the MonoQ profile.

Cwht1p mutants (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,

Table 2 Purification of Cwht1p from transformed *S. cerevisiae* expressing *CWHT1*[a]

Purification step	Total activity (U)[b]	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
70% $(NH_4)_2SO_4$	1,300	9.1	140	100	–
Toyopearl DEAE	830	1.7	490	64	1
FPLC Mono Q HR5/5	350	0.12	2,900	27	6
FPLC His-Trap	160	0.045	3,600	12	7.4

[a] Results are per gram wet weight of yeast cells.
[b] 1 U of activity=1 nmol of released glucose per min

pH 6.8, and concentrated to approximately 0.13 mg protein/ml. 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 synthetic trisaccharide for 30 min and 1 h at 37°C. No activity was detected for mutants at either substrate concentration. No aryl α-glucosidase activity was detected in the 100 mM imidazole fractions for either mutant.

Discussion

Yeast α-glucosidase I (Cwh41p) is a type II ER protein with a proposed domain orientation similar to mammalian α-glucosidase I [4, 5]. However, the function of the predicted domains (i.e. N-terminal and transmembrane) are not well understood. Previously we showed 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 [6]. Moreover, 59 kDa and 37 kDa catalytically active polypeptides were isolated from trypsin proteolysis and endogenous hydrolysis of soluble α-glucosidase I using deoxynojirimycin-based resins [8]. Plasmids carrying truncated forms of *CWH41* (*CWHT1-3*) for encoding polypeptides (Cwht(1–3)p) equivalent to these hydrolytic fragments, fused with C-6xHis, were constructed and transformed into *S. cerevisiae*. Cwht1p (E35-F833) was expressed as a functional polypeptide. However, Cwht2p (R320-F833) and Cwht3p (M526-F833) were not detectable by immunoblot analyses. These results suggest that the region of the *CWH41* gene encoding E35-M526 of Cwh41p is essential for functional expression. It is possible that deletion of this region has an effect on gene expression and/or stability of mRNA.

The specific activity of purified Cwht1p was determined to be 3,600 U/mg protein which is comparable to the specific activity of the pure soluble α-glucosidase I (3,000 U/mg protein). Therefore, M1-E33 of Cwh41p, containing the predicted N-terminal (M1-K10) and the transmembrane domain (T11-I28), is not required for α-glucosidase I folding and activity. It has also been shown that protein chimeras fused with the region containing the predicted N-terminal and transmembrane domain of human α-glucosidase I folded correctly [15, 16]. Also fusion of the yeast enzyme with 6xHis residues at the C-terminal does not alter enzyme conformation as Cwht1p remained fully functional towards the synthetic trisaccharide.

Our 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

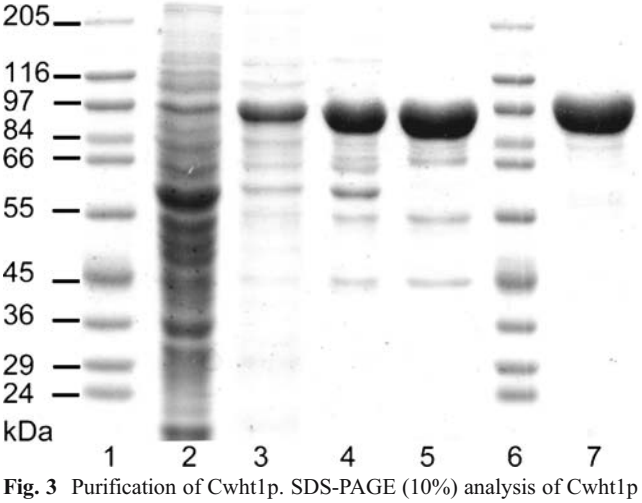

Fig. 3 Purification of Cwht1p. SDS-PAGE (10%) analysis of Cwht1p fractions at different stages of purification. Lane 1 and 6, molecular weight standards; lane 2, 70% ammonium sulfate precipitate; lane 3, 0.2 M NaCl α-glucosidase I active fraction from Toyopearl DEAE; lane 4, 0.3 M NaCl α-glucosidase I active fraction from MonoQ; lane 5, 100 mM imidazole active fraction from His-Trap; lane 7, pure α-glucosidase I from yeast expressing *CWH41*; lane1 and 6, molecular weight standards. Gel was stained by Coomassie blue

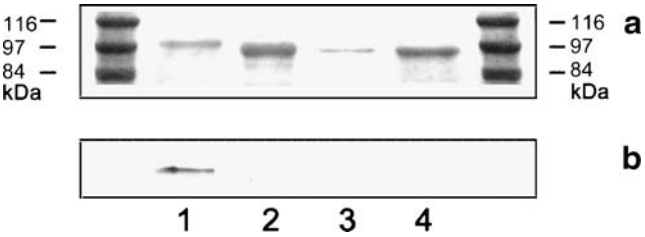

Fig. 4 Cwht1p is not glycosylated. **a** SDS-PAGE (10%) of the soluble α-glucosidase I (lane 1); soluble α-glucosidase I treated with N-glycosidase F (lane 2); Cwht1p (lane 3); Cwht1p treated with N-glycosidase F (lane 4). **b** Western blot analysis of the biotinylated glycoprotein was carried out using anti-biotin polyclonal antibody conjugated with peroxidase and 4-chloro-1-naphthol as the substrate

during translation. The soluble α-glucosidase I activity of yeast expressing *CWHT1* increased 4-fold per g of biomass compared to yeast overexpressing *CWH41*, and immunoblot analysis did not detect any band corresponding to Cwht1p in the microsomal protein fraction. As well, although Cwht1p carries all of five *N*-glycosylation sites of Cwh41p, it was not glycosylated. 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 [15]. However, such a region has not been identified within the predicted N-terminus of yeast α-glucosidase I. 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 [17]. Thus, it is plausible that the hydrophobic region of yeast α-glucosidase I (T11-I28) functions as a signal sequence for translocating the protein into the ER.

Although Cwht1p was not glycosylated, it had the same specific activity as the glycosylated soluble glucosidase I. Studies have also shown that inhibiting *N*-glycosylation of other enzymes that are natively *N*-glycosylated [18–20] did not affect activity and/or conformation during folding. In addition, treatment of pure Cwht1p with trypsin resulted in the release of catalytically active polypeptides similar to those obtained from the glycosylated enzyme [7, 8]. This implies that glycosylation of yeast α-glucosidase I is not critical for the protein to acquire its active conformation through interaction with the ER lectin like chaperones and does not change resistance of the catalytic domain to proteolysis. Therefore, functional expression of the soluble form of yeast α-glucosidase I (Cwht1p) in *E. coli* should be investigated. This may facilitate expression, extraction and purification of the enzyme compared to using *S. cerevisiae*.

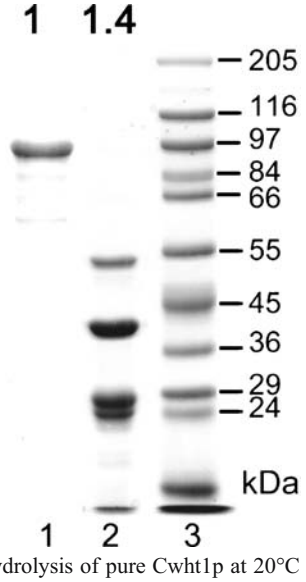

Fig. 5 Trypsin hydrolysis of pure Cwht1p at 20°C releases a mixture of α-glucosidase I active polypeptide(s). Pure Cwht1p was mixed with the modified trypsin (Promega), with the ratio of 8:1 and kept at 20°C for 20 h. Enzyme without trypsin was incubated under the same condition (control). An aliquot 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. Cwht1p only (control, lane 1), hydrolyzed Cwht1p (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 Cwht1p compared to control Cwht1p

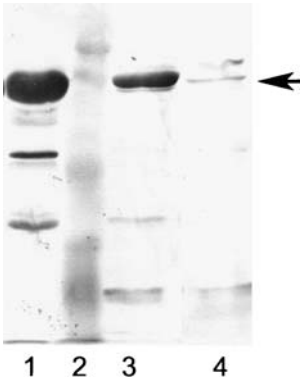

Fig. 6 Western blot analysis of E613A and D617A. 100 mM imidazole IMAC fractions of mutated proteins were run on SDS-PAGE gel (10%) and were electroblotted onto PVDF. Immunoblot was carried out using anti-His monoclonal antibody. Lane 1, Cwht1p; lane 2, prestained molecular weight marker; lane 3, E613A; lane 4, D617A. *Arrow* shows the position of Cwht1p

It is well accepted that the mechanism of action of glycosyl hydrolases involves a pair of carboxylic acid residues [10]. Yeast α-glucosidase I is an inverting enzyme [9] with one carboxylate serving as a general base and the other as a general acid [11]. 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, indicating that they may possess a prominent function in enzyme activity. Indeed, E580A and D584A variants of Cwht1p did not exhibit any detectable glucosidase I activity with the synthetic trisaccharide and α-nitrophenyl α-D-glucopyranoside. These drawbacks prevented acquisition of important kinetic parameters for E580A and D584A. However, other functional roles for these carboxylic acid residues cannot be excluded. For instance, it has been reported that in other inverting glycosidase such as glucoamylase (EC 3.2.1.3, family 15) from *Aspergillus* sp, non-active site carboxylic acid residues such as Asp55 [21, 22] and Glu180 [23] are critical for hydrogen bonding with hydroxyl groups of sugar substrates. It was also reported that site-directed mutagenesis of the non-catalytic Asp101 of α-amylase (EC 3.2.1.2, family 14), resulted in undetectable activity [24]. It was noted that Asp101 interacts with the non-reducing terminus of the substrate, adjusting the position of the sugar for catalysis [25]. It was also shown that

expression of E580A and D584A was drastically decreased compared to Cwht1p hindering further structural analysis. However, Ala is a non-ionizable, sterically conserved replacement for the carboxylic acid residues that should have a low impact on structure, and has been used frequently in site-directed mutagenesis [25]. Nevertheless, the possibility of inactivation of enzyme due to changes in structural conformation associated with site-directed mutagenesis cannot be completely excluded. Results of this study indicate that E613 and D617 of yeast α-glucosidase I are important either for mechanism of action or for establishing the active structural conformation. Therefore, the role of these two residues should be further investigated by substitution with sterically conserved residues such as Asn and Gln. 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 α-glucosidase I.

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References

1. Coutinho, P.M., Henrissat, B.: Carbohydrate-active enzymes, http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html (1999)
2. Kalz-Fuller, B., Bieberich, E., Bause, E.: Cloning and expression of glucosidase I from human hippocampus. Eur. J. Biochem. **231**, 344–351 (1995)
3. Romaniouk, A.V., Silva, A., Feng, J., Vijay, I.K.: Synthesis of a novel photoaffinity derivative of 1-deoxynojirimycin for active site-directed labeling of glucosidase I. Glycobiology **14**, 301–310 (2004)
4. Jiang, B., Sheraton, J., Ram, A.F., Dijkgraaf, G.J., Klis, F.M., Bussey, H.: *CWH41* encodes a novel endoplasmic reticulum membrane N-glycoprotein involved in beta 1,6-glucan assembly. J. Bacteriol. **178**, 1162–1171 (1996)
5. Romero, P.A., Dijkgraaf, G.J., Shahinian, S., Herscovics, A., Bussey, H.: The yeast CWH41 gene encodes glucosidase I. Glycobiology **7**, 997–1004 (1997)
6. Dhanawansa, R., Faridmoayer, A., van der Merwe, G., Li, Y.X., Scaman, C.H.: Overexpression, purification, and partial characterization of *Saccharomyces cerevisiae* processing alpha glucosidase I. Glycobiology **12**, 229–234 (2002)
7. Faridmoayer, A., Scaman, C.H.: An improved purification procedure for soluble processing alpha-glucosidase I from *Saccharomyces cerevisiae* overexpressing *CWH41*. Protein Expr. Purif. **33**, 11–18 (2004)
8. Faridmoayer, A., Scaman, C.H.: Binding residues and catalytic domain of soluble *Saccharomyces cerevisiae* processing alpha-glucosidase I. Glycobiology **15**, 1341–1348 (2005)
9. Palcic, M.M., Scaman, C.H., Otter, A., Szpacenko, A., Romaniouk, A., Li, Y.X., Vijay, I.K.: Processing alpha-glucosidase I is an inverting glycosidase. Glycoconj. J. **16**, 351–355 (1999)
10. Koshland, D.E. Jr.: Stereochemistry and mechanism of enzymatic reactions. Biol. Rev. **28**, 416–436 (1953)
11. Rye, C.S., Withers, S.G.: Glycosidase mechanisms. Curr. Opin. Chem. Biol. **4**, 573–580 (2000)
12. Romaniouk, A., Vijay, I.K.: Structure-function relationships in glucosidase I: amino acids involved in binding the substrate to the enzyme. Glycobiology **7**, 399–404 (1997)
13. Gietz, D., St Jean, A., Woods, R.A., Schiestl, R.H.: Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20**, 1425 (1992)
14. Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M., van Vuuren, H.J.: Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nat. Biotechnol. **15**, 253–257 (1997)
15. Hardt, B., Kalz-Fuller, B., Aparicio, R., Volker, C., Bause, E.: (Arg)3 within the N-terminal domain of glucosidase I contains ER targeting information but is not required absolutely for ER localization. Glycobiology **13**, 159–168 (2003)
16. Tang, B.L., Low, S.H., Hong, W.: Endoplasmic reticulum retention mediated by the transmembrane domain of type II membrane proteins Sec12p and glucosidase 1. Eur. J. Cell Biol. **73**, 98–104 (1997)
17. Martoglio, B., Dobberstein, B.: Signal sequences: more than just greasy peptides. Trends Cell Biol. **8**, 410–415 (1998)
18. Blum, A., Martin, H.J., Maser, E.: Human 11beta-hydroxysteroid dehydrogenase type 1 is enzymatically active in its nonglycosylated form. Biochem. Biophys. Res. Commun. **276**, 428–434 (2000)
19. Kyossev, Z.N., Reeves, W.B.: N-glycosylation is not essential for enzyme activity of 11 beta-hydroxysteroid dehydrogenase type 2. Kidney Int. **52**, 682–686 (1997)
20. Witt, S., Singh, M., Kalisz, H.M.: Structural and kinetic properties of nonglycosylated recombinant *Penicillium amagasakiense* glucose oxidase expressed in *Escherichia coli*. Appl. Environ. Microbiol. **64**, 1405–1411 (1998)
21. Sierks, M.R., Bock, K., Refn, S., Svensson, B.: Active site similarities of glucose dehydrogenase, glucose oxidase, and glucoamylase probed by deoxygenated substrates. Biochemistry **31**, 8972–8977 (1992)
22. Sierks, M.R., Svensson, B.: Functional roles of the invariant aspartic acid 55, tyrosine 306, and aspartic acid 309 in glucoamylase from *Aspergillus awamori* studied by mutagenesis. Biochemistry **32**, 1113–1117 (1993)
23. Sierks, M.R., Svensson, B.: Kinetic identification of a hydrogen bonding pair in the glucoamylase-maltose transition state complex. Protein Eng. **5**, 185–188 (1992)
24. Totsuka, A., Nong, V.H., Kadokawa, H., Kim, C.S., Itoh, Y., Fukazawa, C.: Residues essential for catalytic activity of soybean beta-amylase. Eur. J. Biochem. **221**, 649–654 (1994)
25. Ly, H.D., Withers, S.G.: Mutagenesis of glycosidases. Ann. Rev. Biochem. **68**, 487–522 (1999)