Deciphering the Effect of the Different N-Glycosylation Sites on the Secretion, Activity, and Stability of Cellobiohydrolase I from *Trichoderma reesei*

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N-linked glycosylation modulates and diversifies the structures and functions of the eukaryotic proteome through both intrinsic and extrinsic effects on proteins. We investigated the significance of the three N-linked glycans on the catalytic domain of cellobiohydrolase I (CBH1) from the filamentous fungus *Trichoderma reesei* in its secretion and activity. While the removal of one or two N-glycosylation sites hardly affected the extracellular secretion of CBH1, eliminating all of the glycosylation sites did induce expression of the unfolded protein response (UPR) target genes, and secretion of this CBH1 variant was severely compromised in a calnexin gene deletion strain. Further characterization of the purified CBH1 variants showed that, compared to Asn270, the thermal reactivity of CBH1 was significantly decreased by removal of either Asn45 or Asn384 glycosylation site during the catalyzed hydrolysis of soluble substrate. Combinatorial loss of these two N-linked glycans further exacerbated the temperature-dependent inactivation. In contrast, this thermal labile property was less severe when hydrolyzing insoluble cellulose. Analysis of the structural integrity of CBH1 variants revealed that removal of N-glycosylation at Asn384 had a more pronounced effect on the integrity of regular secondary structure compared to the loss of Asn45 or Asn270. These data implicate differential roles of N-glycosylation modifications in contributing to the stability of specific functional regions of CBH1 and highlight the potential of improving the thermostability of CBH1 by tuning proper interactions between glycans and functional residues.

In eukaryotes, the majority of proteins traversing the secretory pathway are N glycosylated cotranslationally. The oligosaccharyl transferase attaches a conserved triantennary N-glycan precursor ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) to the Asn side chain within some Asn-Xxx-Thr/Ser (where Xxx denotes any amino acid except Pro) sequons during cotranslational translocation of the protein into the endoplasmic reticulum (ER) (1–3). The ability of Asn-linked glycans to extrinsically enhance protein folding and reduce aggregation *in vivo* by directing the protein into the calnexin and/or calreticulin (CNX/CRT) folding cycle has been well established (4). If proper folding cannot be achieved, unfolded protein response (UPR) occurs or glycoproteins are marked for ER-associated degradation, which is also directed by the glycan tag (5–8). N-glycans can also exert effects on protein stabilization and folding acceleration through an apparent intrinsic chemical mechanism (9–13).

The filamentous fungal species *Trichoderma reesei* is famous for its high capacity to secrete large amounts of glycoside hydrolase enzymes that act synergistically to release fermentable sugars from plant cell walls (14). Cellobiohydrolase I (CBH1) belonging to the glycoside hydrolase family 7 (also termed CEL7A) represents the highest secreted cellulase component, typically making up to 60% of the total secreted proteins (15). Many cellulases produced by *T. reesei*, including CBH1, are multimodular with carbohydrate-binding modules and catalytic modules connected by flexible, glycosylated linkers (16). They are also glycoproteins with both O- and N-linked glycosylation sites (17–20). The O-glycosylated linker in CBH1 has been shown to bind cellulose directly and aid in dynamic binding during the enzymatic deconstruction of plant cell walls (21). N-glycosylation of CBH1 occurs at three (Asn45, Asn270, and Asn384) of the four potential N-glycosylation sites in its

catalytic domain (18). While Asn45 is located at the entrance of the catalytic tunnel, Asn270 is located near the distal end of the catalytic tunnel corresponding to the exit for the released cellobiose. Asn384, on the other hand, is located on a peptide loop at the bottom of the enzyme, which partially forms the catalytic tunnel on the loop region (22, 23). The type and extent of N-glycan on the catalytic domain varied from host strains and culture conditions (22, 24–26). The effects of N-glycosylation on heterologously expressed *T. reesei* CBH1 have been studied to some extent (22, 24). When expressed in *Aspergillus niger* var. *awamori*, the purified recombinant CBH1 was found to contain six times the amount of N-linked glycans than the enzyme purified from *T. reesei*. The recombinant CBH1 also displays reduced activity and increased nonproductive binding on cellulose (24). On the other hand, the absence of a glycan at Asn384 results in an improvement in its activity toward cellulose compared to wild-type (WT) recombinant CBH1 (22). Nonetheless, systematic analysis of the N-glycan on the secretion and activity of CBH1 has been lacking (27).

In the present study, we systematically evaluate the role of N-glycosylation in the secretion of CBH1 in *T. reesei* by constructing a series of N-glycosylation defective mutants through eliminating the respective glycosylation sites and expressing the respective

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variants on site in *T. reesei*. We further probe the differential contributions of N-glycosylation sites to the thermal stability and activity of purified CBH1.

MATERIALS AND METHODS

Strains, medium, and cultivations. *Trichoderma reesei* TU-6 (ATCC MYA-256) (28), the uridine auxotroph with a mutant *pyr4* (orotidine 5'-phosphate decarboxylase-encoding) derived from *T. reesei* QM9414 (ATCC 26921), was used as a parental strain throughout this work. *T. reesei* strains were cultivated at 30°C with rotary shaking at 180 rpm in 1-liter flasks containing 250 ml of minimal medium as described by Mandels et al. (29) using glycerol (1% [vol/vol]) or Avicel (1% [wt/vol]) as the sole carbon source. *Escherichia coli* DH5 α was used for plasmid construction and amplification.

In order to analyze cellulase production, *T. reesei* strains were precultured on glycerol for 36 h and transferred to the fresh medium for another 12 h. Mycelia were then collected by filtration and washed using medium without carbon source. Equal amounts of mycelia were transferred to a fresh medium with Avicel (1% [wt/vol]) as the sole carbon source and incubated for the indicated time period.

Disruption of *cbh1* gene in *T. reesei*. To delete the *cbh1* gene, its coding sequence was replaced by the *pyr4* gene. The 2.7-kb *pyr4* fragment was amplified from pFG1 (30) and fused with a fragment 300 bp upstream of the *cbh1* start codon amplified from *T. reesei* genomic DNA by overlap-extension PCR. The fused fragment was digested with XbaI/BamHI and ligated into the same sites of pUC19 to obtain pUC*pyr4*. DNA fragments with ~1.5 kb of 5' and 3' flanking sequences of *cbh1* were amplified from *T. reesei* genomic DNA and successively inserted into pUC*pyr4* to obtain pUC*cbh1pyr4*, which was further digested by SalI and EcoRI to generate the 6-kb deletion cassette. This 6-kb fragment was subsequently used to transform *T. reesei* TU-6 as previously described (31). Transformants were selected on minimal medium for uridine prototrophy. Anchored PCR and Southern blot analysis were performed to verify the correct integration events.

Construction of N-glycosylation mutants. To construct CBH1 N-glycosylation mutant strains, the *pyr4* gene introduced into Δ *cbh1* strain was replaced by the coding sequence of *cbh1* with mutations to eliminate N-glycosylation at the respective site as indicated. Three fragments, including the *trpC* terminator from *Aspergillus nidulans* with a SpeI site at the 5' end, the *gpd1* promoter derived from *T. reesei* (32), and the *hph* gene (hygromycin B phosphotransferase encoding) amplified from pRLMex30 (33) with a BamHI site at the 3' end, were successively fused together by overlap-extension PCR and ligated into pMD19-T (TaKaRa, Japan) digested with SpeI and BamHI to obtain pMD*tph*. Two DNA fragments corresponding to ~1.5 kb of 5' and 3' flanking sequences of *cbh1* were amplified from *T. reesei* genomic DNA and introduced into pMD*tph* sequentially after respective digestion with BamHI/SmaI and SphI/SalI to generate pMD*up-tph-down*. The open reading frame of *cbh1* amplified from *T. reesei* genomic DNA was fused at the 3' end with a sequence encoding three copies of hemagglutinin (HA) tag and ligated to pMD*up-tph-down* after digestion with SpeI and SalI to generate pMD*up-WT-tph-down*. All of the *cbh1* fragments with targeted mutations to eliminate N-glycosylation by replacing asparagine with glutamine were generated by site-directed mutagenesis using overlap-extension PCR with pMD*up-WT-tph-down* as the template and ligated to pMD*up-tph-down* to obtain the respective constructs for expressing CBH1 variants. These constructs with verified mutations by DNA sequencing were digested with SphI and SmaI to release the 7.5-kb fragments, which were subsequently used to transform the Δ *cbh1* strain to generate *T. reesei* WT and variant strains, including M45, M270, M384, MD1, MD2, and MT. Transformants were selected on plates containing hygromycin (120 μ g/ml) and 5-fluoroorotic acid (1.2 mg/ml). Anchored PCR and DNA sequencing were conducted to verify the correct integration events. Oligonucleotides, including gene-specific primers used here for plas-

mid constructions, gene deletions, or quantitative reverse transcription-PCR (RT-PCR), are listed in Table 1.

Disruption of *cnl1* gene in *T. reesei*. To delete the *cnl1* gene, its coding sequence was replaced by the *pyr4* gene. Two DNA fragments around 2 kb upstream and downstream of the *cnl1* gene were amplified from *T. reesei* genomic DNA and ligated into pDONOR*pyr4* (34) by BP-cloning to obtain the disruption vector pDEL*cnl1*, which was linearized with I-SceI and used to transform *T. reesei* WT and MT strains constructed as described above, respectively. Transformants were selected on minimal medium for uridine prototrophy.

Nucleic acid isolation and Southern blotting. Fungal mycelia were collected by filtration, washed with water, and frozen immediately in liquid nitrogen. Fungal genomic DNA was extracted by using an EZNA Fungal DNA miniprep kit (Omega Biotek, Doraville, GA) according to the manufacturer's protocol. Southern blotting was performed as previously described (31).

Purification of CBH1 proteins. One liter of culture supernatant of *T. reesei* was centrifuged at 4°C for 10 min at 11,000 rpm to remove impurities and mycelial debris. The supernatant was further concentrated 50-fold by ammonium sulfate precipitation. The precipitate was dissolved in 20 mM citric acid-sodium citrate buffer (pH 6.0) and centrifuged at 4°C for 15 min at 11,000 rpm to remove insoluble particles. After filtration through a 0.22- μ m-pore-size polypropylene membrane, the supernatant was loaded onto a Sephadex G-25 (GE Healthcare, Sweden) column, which was equilibrated with 20 mM citric acid-sodium citrate buffer (pH 6.0) at a flow rate of 1 ml/min. The main fractions eluted from this column were collected and subsequently applied to a DEAE Sepharose Fast Flow (GE Healthcare) column (XK16; Pharmacia, Sweden) equilibrated with 20 mM citric acid-sodium citrate buffer (pH 6.0) at a flow rate of 1 ml/min. The bound fraction was eluted stepwise with buffers containing increasing concentrations of NaCl up to 1.0 M. Fractions containing CBH1 proteins verified by Western blotting were pooled and buffer exchanged to 100 mM sodium acetate buffer (pH 3.6) by repeated ultrafiltration using Amicon Ultra-15 centrifugal filter units (Millipore Corp., Ireland) with a 10-kDa cutoff. The buffer-exchanged proteins were further loaded onto a Mono Q 5/50 GL column (GE Healthcare) equilibrated with 100 mM sodium acetate buffer (pH 3.6), and this column was eluted with a linear gradient elution of sodium acetate buffer at pH 3.6 (100 to 250 mM) at a flow rate of 1 ml/min. Fractions containing CBH1 proteins were divided into aliquots and frozen at -80°C.

Quantitative RT-PCR. Total RNA used was extracted and purified using a Qiagen RNeasy plant minikit (Qiagen, Germany) according to the instructions. Reverse transcription was performed with the PrimeScript RT reagent kit (TaKaRa, Japan). Quantitative RT-PCR was performed on a Bio-Rad myIQ2 thermocycler (Bio-Rad, USA). Amplification reactions and data analysis using the relative quantitation/comparative C_T ($\Delta\Delta C_T$) method were carried out as described previously (34).

Protein analysis. SDS-PAGE and Western blotting were carried out according to standard protocols (35). Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard (36). Detection of CBH1 was conducted by immunoblotting using a polyclonal antibody raised against amino acids (426 to 446) of CBH1 as previously described (31).

Enzyme activity measurement. The enzyme activity of CBH1 was measured using *p*-nitrophenol-D-cellobioside (pNPC) (Sigma, USA) and Avicel (Sigma) as the substrates, respectively. The pNPC hydrolytic activity was determined by measuring the amount of pNP released from pNPC as described by Deshpande et al. (37). One unit of pNPC hydrolytic activity is defined as the amount of enzyme that liberates 1 nmol of pNP per min. Lineweaver-Burk plot analysis was used to calculate the kinetic parameters K_m and k_{cat} (38). Measurement of Avicel hydrolytic activity was performed in 500 μ l of 50 mM sodium acetate buffer (pH 5.0) containing 1% Avicel and purified CBH1 proteins and incubated at various temperatures, as indicated with a shaking rate of 100 rpm. Dinitrosalicylic acid

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5'-3') ^a
<i>Δcbh1</i> Up F	GCGTCGACGTTGTGAAGTCGGTAATCCCGCTG (Sall)
<i>Δcbh1</i> Up R	GCTCTAGACGGTTGACTATTGGGTTTCTGTGCC (XbaI)
<i>Δcbh1 pyr4</i> F	GCTCTAGACTGCAGGTCAACGGATCTGTGTC (XbaI)
<i>Δcbh1</i> DR F	GGTAGGATGGTTTGGATGCAGTTGCAAATGCAAAGTGTGGTAGGATCG
<i>Δcbh1</i> DR R	CGGGATCCCGGTTGACTATTGGGTTTCTGTGCC (BamHI)
<i>Δcbh1</i> Down F	CGGGATCCAGCCTGACGCACCGGTAGATTCTTG (BamHI)
<i>Δcbh1</i> Down R	CGGAATTCCTTCTTAACACTTCGGTGGAGGTGTC (EcoRI)
<i>Δcbh1</i> Up AnF	TGTTTCGGCTACGGTGAAGAACTG
<i>pyr4</i> 344R	TGAAGCAGAAATAGGGTGAATGATA
<i>pyr4</i> 2597F	TATTTTGTATTGGCATTGCTTTT
<i>Δcbh1</i> Down AnR	CTTATTGAGACCATGCGTGCTAAT
<i>Δcbh1</i> ORF F	ACTCAACAGACAGGCTCCGTGGTC
<i>Δcbh1</i> ORF R	AGATTCGACCTGAGCAGGGACACC
WT up F	ACATGCATGCTTGTGAAGTCGGTAATCCCGCTGTA (SphI)
WT up R	ACGCGTCGACCGAGTCCGCGGTTGACTATTGGGT (Sall)
WT ORF HA F	GAACCCTTACTACTCTCAGTGCCTGATAAACCGCTCCGGTTCTGCTG
WT ORF HA R	CTAGACTAGT GTGAATGATCGTTCCACTTTTATAGC (SpeI)
WT ORF F	ACGCGTCGACATGTATCGGAAGTTGGCCGTCAT (Sall)
WT <i>trpC</i> F	CTAGACTAGTCGATCCACTTAACGTTACTGAAATCA (SpeI)
WT <i>trpC</i> R	GCCATATTGATGTAAGGTAGCTCTCTCGAGTGGAGATGTGGAGTGG
WT <i>hph</i> F	GAGAGCTACCTTACATCAATATGGC
WT <i>hph</i> R	CGGTGAGTTCAGGCTTTTTCATTTTGTATCTGCGAATTGAGCTTGC
WT <i>gpd1</i> R	CTATTCCTTTGCCCTCGGACG
WT down F	CGCGGATCCAGCTCCGTGGCGAAAGCCTGA (BamHI)
WT down R	TCCCCCGGGCCATTCTTAACACTTCGGTGGAGGT (SmaI)
WT An <i>trpC</i> R	CTAGAAGGCACTCTTTGCTGCTTG
WT An <i>hph</i> F	TATCAGAGCTTGGTTGACGGCAAT
M45R	CATCGTAGCAGTTCGTGCTGCTTTGCGTAGCGTGAGTCCAGCGCC
M270R	CCAGGGCGGTAGAAGCTGGTTTGGCCAGGCGGTATGGGTTCC
M384F	GCTGGACTCCACCTACCCGACACAAGAGACCTCCTCCACACCCGGT
<i>cne1</i> up F	TAGGGATAACAGGGTAATGCTTTGTAAAGCCACACCTAGTCCTG
<i>cne1</i> up R	GGGGACAAGTTTGTACAAAAAGCAGGCTAAAAGTTCAACGTTTTGCAGCAGTAAG
<i>cne1</i> down F	GGGGACCACTTTGTACAAAGAAAGCTGGGTAATGATTACTCTAAAGCAACCGTAACCT
<i>cne1</i> down R	ATTACCCTGTTATCCCTAGTCTCCATGTCCTTGGACACCTC
<i>cne1</i> UA F	CTCTACCAGGACCAGCTCTGCCTCT
<i>cne1</i> DA F	GCCATTGCCCCTTTAGTGAGTTTC
<i>cne1</i> ORF F	TCCACCTTTACTGTGAGTTTGCC
<i>cne1</i> ORF R	GGAACGAACTTACAGCTCCCATG
qRT-CBH1 F	CTTGGCAACGAGTTCTCTT
qRT-CBH1 R	TGTTGGTGGGATACTTGCT
qRT-ACT F	TCCATCATGAAGTGCGAC
qRT-ACT R	GTAGAAGGAGCAAGAGCAGTG
<i>bip</i> RT1 F	CGACATCAAGCACTTCCCCTAC C
<i>bip</i> RT1 R	CTTGTCAGACCATAGGCGATA
<i>pdi</i> RT2 F	CGGCAACATCAACCTCAAGACC
<i>pdi</i> RT2 R	GTAAGAGTGGGCAACGACAACG
<i>cne</i> RT1 F	GAGGACAAGACGCCCCGCAACT
<i>cne</i> RT1 R	CCTCGCAAGCGCCTTCTCAAT

^a The restriction enzyme sites used are shown as underlined sequences. The restriction enzymes are indicated in parentheses.

(DNS) method was performed to determine the amount of cellobiose released from Avicel (39).

DSC. Thermal unfolding of CBH1 proteins was measured by using a Microcal VP-Capillary differential scanning calorimetry (DSC; Microcal Northampton, MA) at a scan rate of 60°C/h. Thermograms were gathered from purified CBH1 protein samples in 20 mM sodium acetate buffer at pH 5.0 with data analyzed by the Origin software (Microcal, Sweden).

Far-UV CD spectroscopy. Circular dichroism (CD) spectra of CBH1 proteins with the same concentration (0.12 mg/ml) with or without incubation at 62°C for indicated time periods were collected from 260 to 190

nm at a scan speed of 200 nm/min with a bandwidth of 2 nm on a Jasco J-810 spectropolarimeter (Jasco, Japan).

RESULTS

The absence of N-glycosylation of CBH1 has little effect on its extracellular secretion. Structural examination of the glycosylation motifs showed the placement of all three N-linked glycosylation sites on the surface of CBH1, whereas the catalytic site is within an enclosed tunnel running along the concave face of a β -sandwich packed mostly with hydrophobic side chains

FIG 1 Structure of the catalytic domain of Cel7A with labeled glycosylation sites. The cellulose chain bound in the tunnel is shown as a cyan stick model. The cellobiose unit is shown as an orange stick model. The structure was simulated and displayed using PyMOL (DeLano Scientific [<http://www.pymol.org>]).

(Fig. 1). In order to investigate the role of N-linked glycosylation in CBH1, single-site N-glycosylation mutants (M45, M270, and M384) and double-site mutants (M45M270 [here called MD1] and M45M384 [referred to here as MD2]), as well as the triple-site mutant (MT), were constructed by individual or combinatorial targeted change of asparagine at sites 45, 270, and 384 to the almost similar glutamine to minimize any potential perturbation on the structure of CBH1. Expression of all of the CBH1 variants was directed by the *cbh1* promoter by replacing the endogenous *cbh1* locus with wild-type (WT) and mutant *cbh1* with three copies of HA tagged at the C terminus. The presence of HA tags was found to have no significant effect on the secretion and activity of recombinant WT CBH1 compared to the endogenous CBH1 from TU-6 (data not shown). Measurement of extracellular protein concentration and pNPC hydrolytic activity of the culture supernatant from the mutant and the WT strains with Avicel as the sole carbon source showed that elimination of N-linked glycosylation at either single or double or triple sites did not significantly affect the extracellular total protein production and exoglucanase activity (Fig. 2A and B). Further Western blot analysis of CBH1 secreted into the culture supernatant from these mutant and WT strains indicated that the absence of N-glycosylation hardly affected the extracellular level of CBH1 except that a marginal difference between the production of WT and mutated CBH1s at earlier time points was observed (Fig. 2C). Coomassie blue staining of the same SDS-PAGE also revealed roughly the same levels of total extracellular proteins with induction between WT and mutant strains (data not shown).

Elimination of N-glycosylation in CBH1 induces expression of the UPR target genes. Although the absence of N-glycosylation had little effect on the secretion of CBH1, we wondered whether the expression of UPR target genes is modulated to adapt to the folding of unglycosylated CBH1. The transcript levels of major ER chaperones and foldases, *cne1* (Tr_73678) encoding the *T. reesei* homolog of calnexin, *pdi1* (Tr_122415) encoding protein disulfide isomerase, and *bip1* (Tr_122920) encoding the major ER chaperone were analyzed for strains expressing WT and MT

CBH1 cultured with Avicel. Although the induced level of MT *cbh1* transcript was slightly lower than that of WT, the mRNA levels of *bip1*, *cne1*, and *pdi1* in strain expressing MT CBH1 were increased concomitantly with the expression of *cbh1* gene (Fig. 3A). The mRNA levels of *cne1*, *bip1*, and *pdi1* at 24 h were approximately 5-, 4-, and 2-fold, respectively, that expressing WT CBH1. To further test whether the induced expression of the UPR target genes is involved in the folding and thus the efficient secretion of nonglycosylated CBH1, we deleted *cne1* in the WT and MT strains, respectively. As shown in Fig. 3B, although the deletion of *cne1* had hardly any effect on the secretion of WT CBH1, its absence drastically compromised the secretion of MT CBH1. Together, these results indicate that N-glycosylation may not directly participate in the chaperone-mediated folding of CBH1, but its presence does contribute to the folding process.

The absence of N-glycosylation in CBH1 does not significantly decrease its catalytic activity. As described above, the absence of N-glycosylation in CBH1 hardly affected its extracellular secretion level, we further purified the respective WT and mutant CBH1 proteins to analyze whether their exoglucanase activity is affected (Fig. 4A). The kinetic constants (k_{cat} and K_m) of the seven enzyme variants (WT, M45, M270, M384, MD1, MD2, and MT) with a soluble substrate, pNPC, were measured at 45°C, and the results are shown in Table 2. There is no significant difference in these detected values, indicating that the absence of N-glycosylation does not influence its catalytic activity or binding affinity on pNPC. In order to study the hydrolysis of insoluble cellulose, we used crystalline cellulose Avicel as a substrate and followed the process of cellobiose production (Fig. 4B). Although a similar slight decrease in the velocities of cellobiose production was observed for variants containing Asn45 and Asn384, the hydrolytic activity of all mutants was largely unaffected. Taken together, the absence of N-linked glycosylation of purified CBH1 does not significantly influence its catalytic activity with both soluble and insoluble substrates.

N-glycosylation at Asn384 and Asn45 is important for the thermal reactivity of CBH1. Next we analyzed the influence of the absence of N-glycosylation on the stability of purified CBH1 by measuring its thermal transition temperature using DSC. As also shown in Table 2, CBH1 variants of M384, double-site mutant MD2, and triple-site mutant MT had a thermal transition temperature ~2°C lower than that of WT CBH1. In contrast, there was no significant difference in the thermal transition temperatures between WT and CBH1 variants M45, M270, or MD1. These data indicate that the absence of N-glycosylation at Asn384 influences the thermal stability of CBH1.

To evaluate the effect of differences in the thermal transition temperature on the hydrolytic property of CBH1, catalytic performances of CBH1 variants at temperatures higher than 45°C were analyzed with pNPC as a substrate. Although both WT and MT CBH1 proteins displayed the highest hydrolytic activity at 60°C, the activity of MT CBH1 dropped dramatically when the temperature was increased to values right above 60°C, compared to the slight decrease for WT at 62°C (Fig. 5A). Correspondingly, when preincubated at 62°C, inactivation of MT, MD2, and M384 was observed to occur at a faster rate than that of WT, and the hydrolytic activities toward pNPC were almost abolished, whereas that of WT and M270 remained 50% of the original activity after incubation for 1 h. The activity of M45 and MD1 was intermediate between WT and MT after the same treatment (Fig. 5B). To probe

FIG 2 The absence of N-glycosylation of CBH1 hardly affects its extracellular secretion. (A) Extracellular protein concentration of strains expressing WT and mutant CBH1. (B) pNPC hydrolytic activity of the culture supernatant for strains expressing CBH1 variants after 60 and 80 h of induction with cellulose. The values in panels A and B are means of three biological replicates. Error bars show the standard deviations of the mean. (C) Western blot analysis of CBH1 secreted into the culture supernatant from mutant and WT strains after induction with 1% Avicel. Equal amount of culture supernatant relative to biomass was loaded for all strains.

further into the relative contributions of different N-glycosylation sites to the thermal stabilizing effect, we determined the catalytic performance of other CBH1 variants at 62°C (Fig. 5C). In comparison to WT CBH1, mutation at either Asn45 or Asn384 caused approximately 2- and 3-fold reductions, respectively, in terms of pNP production, whereas catalyzed hydrolysis by M270 was only slightly affected. Combinatorial mutations of Asn45 and Asn384 in MD2 and MT CBH1 displayed a negligible hydrolysis compared to WT CBH1, indicating that N-glycosylation at these two sites independently contributes to the thermal stability of CBH1. In contrast to pNPC, MT was still capable of hydrolyzing Avicel at 62°C though mutations at Asn45 and Asn384 resulted in a 1.5-fold less efficient hydrolysis in terms of cellobiose production with the velocity of cellobiose production by MT being reduced to ca. 70% of the WT (Fig. 5D). Instead, preincubating MT at 62°C for 25 min

without Avicel almost abolished the following hydrolysis (Fig. 5E). These results indicate that, although the absence of N-glycosylation results in a destabilizing effect on CBH1, conformation of nonglycosylated CBH1 may be stabilized once bound to insoluble cellulose.

Differential influences of N-glycosylation at Asn384 and Asn45 on the structural stability of CBH1. To investigate the nature of structural changes during the thermal unfolding transition of CBH1 resulted from the absence of N-linked glycosylation, far-UV CD spectroscopy was performed to analyze the structural integrity of CBH1 variants. The general shape of the spectrum of all of the CBH1 variants at 25°C, with negative maxima near 210 nm characteristic of β -sheet secondary structures, was comparable to what has been reported for CBH1 (Fig. 6A) (40). After incubation at 62°C for 10 min, a slight decrease in the ellipticity at

FIG 3 Elimination of N-linked glycosylation in CBH1 induces expression of the UPR target genes. (A) Quantitative RT-PCR analysis of *cbh1*, *bip1*, *pdi1*, and *cne1* gene expression in WT and CBH1 variant strains after induction with 1% (wt/vol) Avicel for different time periods. The values are means of three independent experiments. Error bars are the standard deviations of the mean. (B) Western blot analysis of CBH1 secreted into the culture supernatant from wild-type and $\Delta cne1$ strains expressing WT and MT CBH1, respectively, after induction with 1% Avicel. Equal amounts of culture supernatant relative to biomass were loaded for all strains.

210 nm for M45 and MD1 was observed compared to that of WT and M270. In contrast, the spectral profiles were drastically altered for M384, MD2 and MT with a loss of the minimum characteristic at 210 nm (Fig. 6B). These results indicate that the thermal labile property of N-glycosylation-deficient CBH1 variants results from a loss of regular secondary structure with the absence of N-linked glycan at Asn384 conferring a more pronounced effect than mutation at Asn45 upon temperature elevation. Unlike temperature, the elimination of N-linked glycans did not lead to any alterations in the spectrum at a pH higher than the optimal level (Fig. 6C). These data demonstrate a differential and temperature-specific influence of N-linked glycans on the structural stability of CBH1.

DISCUSSION

Although it is clear that certain N-linked glycans contribute to the folding and stabilization of glycoproteins, our results demonstrate that elimination of the three glycosylation sites does not significantly compromise the efficient secretion of active CBH1. Our results further highlight the influence of the N-linked glycans lo-

cated at the base of the catalytic tunnel and those at the entrance of it on the thermal stability of CBH1. In particular, compared to glycosylation at Asn45, the thermal stabilizing effect conferred by glycosylation at Asn384 seems to result from its contribution to maintaining the secondary structure of the enzyme.

Soluble calreticulin and membrane-bound calnexin have been shown to bind monoglucosylated N-oligosaccharides on newly synthesized glycoproteins via their globular lectin domain. In this way, they promote proper folding by preventing aggregation and by providing a suitable environment in which the associated ERp57 enzyme catalyzes thiol oxidation and isomerization (41, 42). Cnx and Crt also interact with many folding glycoproteins through polypeptide-based interactions, and suppression of the aggregation of nonglycosylated substrates has been reported (43–45). Our results that CBH1 without any N-glycan is secreted almost as efficiently as WT CBH1, suggest that N-glycan mediated extrinsic enhancement of protein folding by recruiting lectin-type chaperones may not play a major role in the folding process of CBH1. The observation that the absence of Cnx in *T. reesei* has no

FIG 4 Avicel hydrolysis by purified WT and mutant CBH1 proteins. (A) SDS-PAGE analysis of purified CBH1 variant proteins. Lanes 1 to 7 correspond to WT, M45, M270, M384, MD1, MD2, and MT CBH1, respectively. (B) Cellobiose production velocity curves of CBH1 variants. Avicel hydrolysis was carried out at 50°C and pH 5.0 with 1 μmol of purified WT and mutant CBH1 proteins. Duplicate assays were performed at each time point. Soluble products were determined by the DNS method using cellobiose as a standard as described in Materials and Methods. The velocities were calculated using origin7.5 (OriginLab, USA) and fitted to the Michaelis-Menten equation.

effect on the secretion of CBH1 further corroborates this assumption. The possibility cannot be excluded that the absence of calnexin, although interfering with Ca²⁺ homeostasis in the ER, does not inflict a significant effect on the proper folding of WT CBH1 if other unknown lectin-type chaperones are otherwise involved (46, 47). On the other hand, elimination of all N-linked glycosylation sites in CBH1 does result in a higher-level expression of some folding chaperone genes. Therefore, one possible explanation is that although N-glycosylation is not necessary for CBH1 folding and efficient secretion considering the existence of other highly capable folding machineries in *T. reesei* (48), the occurrence of N-glycosylation on CBH1 provides it with a folding en-

ergetic advantage. This intrinsic enhancement of folding energetics conferred by the N-glycan structure may further contribute to its stability either by accelerating folding and/or by slowing unfolding. Indeed, our observation that the absence of Cnx in *T. reesei* severely affected the otherwise efficient secretion of MT CBH1 supports such a mechanism.

Many lines of evidence have been presented that glycoproteins are more stable than their corresponding unglycosylated counterparts, even when there are no major structural changes associated with glycosylation (12). The stabilizing influence may largely arise from entropic rather than enthalpic effects by reducing the disorder of the unfolded protein and enhancing the reversibility of the unfolding process (49, 50). On the other hand, N-linked glycans may also facilitate the formation of a key segment of secondary structure, serving to enhance the overall stability of the protein or to enable some specific function performed around the attached site. In both cases, protein-carbohydrate contacts may occur (12). It has been previously shown that heterologously expressed N384A mutant of *T. reesei* cellobiohydrolase I has a lower thermal transition temperature value but displays a 70% greater activity than that of WT CBH1. Unfortunately, N45A mutant was not successfully expressed for characterization (22). In the present study, using an endogenous expression strategy, we showed that, removal of either Asn45 or Asn384 yields a mutant with a thermal labile property in catalyzing soluble substrate. The observed thermal instability of glycosylation deficient mutants is much less significant when catalyzing insoluble cellulose, suggesting that conforma-

TABLE 2 Kinetic constants (K_m and k_{cat}) measured on pNPC (45°C, pH 4.8) and thermal transition temperatures of CBH1 variants^a

Enzyme variant	Mean ± SD		
	K_m (mM)	k_{cat} (min ⁻¹)	T_m (°C) ^b
WT	0.363 ± 0.011	1.178 ± 0.018	65.94 ± 0.085
M45	0.369 ± 0.023	1.068 ± 0.025	65.79 ± 0.042
M270	0.352 ± 0.053	1.066 ± 0.011	65.99 ± 0.014
M384	0.426 ± 0.037	1.204 ± 0.043	63.70 ± 0.002
MD1	0.378 ± 0.026	0.985 ± 0.015	65.37 ± 0.417
MD2	0.359 ± 0.007	1.093 ± 0.025	63.17 ± 0.163
MT	0.434 ± 0.010	1.068 ± 0.008	63.54 ± 0.042

^a All values are means of three independent experiments.
^b Thermal transition temperatures (T_m) were measured by VP-DSC from purified proteins at pH 5.0. Data were analyzed by using Origin for DSC software.

FIG 5 N-glycosylations at Asn384 and Asn45 are important for the thermal reactivity of CBH1. (A) pNPC hydrolytic activity of purified WT and MT CBH1 at different temperatures. (B) Thermal stability of CBH1 variants at 62°C. (C) Progress curves of pNPC hydrolysis by CBH1 variants at 62°C. (D) Product (cellobiose) velocity curves of CBH1 variants. Avicel hydrolysis was carried out as Fig. 4B except the reaction was allowed to occur at 62°C. (E) Hydrolytic activity of WT and MT CBH1 preincubated at 62°C for 25 min without Avicel. The preincubated proteins were then added to the reaction mixture with Avicel, and the cellobiose released was determined after 2 and 4 h of incubation.

tion of the bound unglycosylated enzyme is more resistant to thermal inactivation. Compared to removal of N-glycosylation at Asn384, we cannot distinguish whether mutation at Asn45 also results in the collapse of the tertiary structure under the test conditions, although it could be anticipated that its absence may facilitate the formation of molten globule-like states that otherwise affect the activity (51). In contrast to the previous report (22), we did not find an improved activity for Asn384 mutant toward Avicel. The discrepancy may lie in the facts that the mutant enzyme was expressed homologously rather than heterologously and that asparagine was changed to glutamine instead of alanine in the present study. Considering the relative positions of Asn45 and Asn384 along the catalytic tunnel, an assumption can be made that attachment of an N-

glycan at Asn45 site would stabilize the substrate entrance region, including W40, which is key in recruiting individual substrate chains into the active channel to initiate processive hydrolysis (52). As for Asn384, glycosylation at this site seems to restrain the flexibility of the loop to which it is attached and may thus affect the binding mode of cellobiose relative to the exit tunnel (53). Upon temperature elevation, larger fluctuations of the loop may disturb the neighboring secondary structures and eventually their disassembly. In both cases, it is intriguing to speculate that during the molecular evolution of CBH1, introduction of an N-glycosylation site may compensate for the selection of residues that are required for function but may not be optimal for stability. Directed evolution of residues around these glycosylation sites or modifying glycosylation mo-

FIG 6 Effect of temperature and pH on the secondary structure of CBH1 variants monitored by far-UV CD spectroscopy. (A) Spectral file of CBH1 variants at 25°C. (B) Spectral file of CBH1 variants preincubated at 62°C for 10 min. (C) CD spectra of WT and MT CBH1 at pH 4.8 and 8.0.

tifs at native sites to improve the interactions between N-glycans and functional residues thus represents a potential strategy to enhance enzyme stability while maintaining its catalytic efficiency.

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