Promotion of Extracellular Activity of Cellobiohydrolase I from *Trichoderma reesei* by Protein Glycosylation Engineering in *Saccharomyces cerevisiae*

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**Abstract**

The N-glycosylation in *Saccharomyces cerevisiae* is of the high-mannose type, which affects the activity of the secreted heterologous glycoproteins. Cellobiohydrolase I (Tr-Cel7A) from *Trichoderma reesei*, is thus hyperglycosylated when expressed in *S. cerevisiae*. In the present work, three genes encoding the endogenous mannosyltransferases, Och1p, Mnn9p and Mnn1p, involved in glycoprotein processing in the *S. cerevisiae* Golgi apparatus, were individually or combinatorially disrupted to investigate the effect of the glycosylation extent on the activity of the secreted Tr-Cel7A. The glycosylation of the recombinant Tr-Cel7A was decreased and its extracellular activity was increased in all the deletion mutants. The simultaneous deletion of och1 and mnn1 has the most improvement on extracellular Tr-Cel7A activity. After expressed the α-1,2-mannosidase (Tr-Mds1p) from *T. reesei* in mnn10/och1ΔΔ strain, the Tr-Cel7A activity was further increased up to 320 ± 8% higher than that of the wild type strain. Such activity improvement was due not only to the higher secretion yield but also to the increased specific activity resulted from the changes in glycosylation. The results thus indicated that protein glycosylation engineering in *S. cerevisiae* was an effective approach to improve the extracellular activity of Tr-Cel7A.

Keywords: Cel7A; Mannosyltransferase; α-1,2-Mannosidase; Glycosylation; Protein secretion; Budding yeast

Introduction

The suitable glycosylation in secretory pathway is crucial for the correct folding, stability, bioactivity and extracellular activity of secreted heterologous proteins [1]. One of the advantages of using budding yeast expression system for the production of heterologous glycoproteins is their capability to perform glycosylation, a process that does not occur in *Escherichia coli* [2]. In *Saccharomyces cerevisiae*, glycoprotein are normally modified by the core structure (Man₉GlcNAc₂) which contains three α-1,2 mannosyl linkages in the endoplasmic reticulum (ER). One of α-1,2 mannosyl linkages is hydrolyzed by the site-specific α-1,2-mannosidase Mns1p just before the glycoprotein leaves for the Golgi apparatus. The extension and embanchment of N-linked glycan chains on the newly formed Man₉GlcNAc₂ are performed by the α-1,6-mannosyltransferases such as Och1p, Mnn9p and Mnn1p, and the α-1,2 or α-1,3-mannosyltransferases such as Alg1p and Mnn1p [3] in Golgi apparatus. The mannose residues can reach to about 200 to form a complex mannan [4], which has a potential to affect the activity of N-glycosylated glycoprotein including the heterologous proteins expressed in *S. cerevisiae* [5]. Wang et al. [6] screened *S. cerevisiae* gene-knockout strains of glycosylation-related genes for improved extracellular activity of a heterologous exocellulase (PCX) from the cellulose digesting fungus *Phanerochaete chrysosporium*. They found that the degree of N-glycosylation plays an important role in heterologous cellulase activity and both over- and under-glycosylation may alter the enzyme activity of cellulases in *S. cerevisiae*. Expression of cellulases in the traditional ethanol production strain *S. cerevisiae* is one research aspect of the ethanol production from lignocelluloses using the consolidated bioprocessing (CBP) strategy [7,8]. Among the cellulase system in *Trichoderma reesei*, cellobiohydrolase I (Tr-Cel7A) is recognized as one of the effective enzymes for lignocellulose degradation. However, the Tr-Cel7A was generally hyperglycosylated with lower secretion in *S. cerevisiae* compared with it in *T. reesei* [9-11].

The native Tr-Cel7A in *T. reesei* is a glycoprotein with molecular mass about 70 kDa [10], which is larger than its molecular mass (~45.97 kDa) from the amino acid [12]. A glycosyl chain unit modified on Tr-Cel7A is Man₉GlcNAc₂ formed from the Man₉GlcNAc₂ a same core structure as it in *S. cerevisiae* [13,14]. But during its secretion process, unlike in *S. cerevisiae*, all of the three α-1, 2-mannosyl residues are digested by α-1,2-mannosidase (Tr-Mds1p) in the ER and/or outside of the cell in *T. reesei* [15-17].

In the present work, the glycosylation engineering including the disruption of *S. cerevisiae* endogenous mannosyltransferases and the expression of the Tr-Cel7A α-1,2-mannosidase, was performed. The Tr-Cel7A specific activity and protein secretion yield in yeast were detected to investigate the effect of the glycosylation close to the natural state on its extracellular activity.

Materials and Methods

Strain construction:

Three disruption cassettes containing homologous recombinant arms, MNN1F-loxP-KanMX4-loxP-MNN1R, MNN9F-loxP-KanMX4-loxP-MNN10R and OCH1F-loxP-KanMX4-loxP-OCH1R, were transformed independently or as two successively into the *S. cerevisiae* strain CEN.PK102-3A [18]. The KanMX4 marker was removed using...
the Cre-loxP system as previously described [19], resulting in the single-deletion strains mnn1Δ, mnn9Δ and och1Δ, and the double-deletion strains mnn1Δ/mnn9Δ and mnn1Δ/och1Δ.

The open reading frame (ORF) with its native signal sequence of *T. reesei* α-1,2-mannosidase gene (Tr-MDS1, GenBank accession no. AF212153.1) was amplified from pAJ401mds1 [15] (BCCM/LMBP Plasmid Collection) and ligated into the plasmid pYX242 (Novagen, Madison, WI), which contains the *TP1* promoter and HA tag coding sequence in the 3' end. The resulting plasmid pYX242MH (Figure 1) was then transformed respectively into the wild type and the gene deletion strains. The Tr-cel7A gene with a FLAG tag expressing plasmid pJCF [11] was introduced into all modified strains. All *S. cerevisiae* strains and plasmids used in the present work are listed in Table 1, and the primers used for PCR amplification in the present work are listed in Table S1.

![Figure 1: The physical map of plasmid pYX242MH.](image)

**Culture conditions:**

SC-Ura (1.7 g l\(^{-1}\) yeast nitrogen base, 5 g l\(^{-1}\) ammonium sulfate, and 0.77 g l\(^{-1}\) amino acid mixture with omitted uracil) and SC-Leu-Ura (1.7 g l\(^{-1}\) yeast nitrogen base, 5 g l\(^{-1}\) ammonium sulfate, 0.65 g l\(^{-1}\) amino acid mixture with omitted uracil, leucine and histidine, and 20 mg l\(^{-1}\) histidine) media were used for culture of the recombinant strains. Cultures were maintained at 30 °C for 48 h with shaking at the agitation speed of 200 rpm.

**Purification of Tr-Cel7A from recombinant *S. cerevisiae*:**

The *S. cerevisiae* strain expressing Tr-Cel7A was cultured in SC-Ura medium in 3 L at 30°C. After 48 h, the cells were separated from the growth medium by centrifugation (15 min at 10,000 × g) and filtration over a 0.22 μm pore size. The cell-free culture supernatants were concentrated approximately 100-fold using an ultrafiltration device ÄKTAcross flow automated filtration system (GE HealthCare, USA), equipped with 30 kDa cutoff membranes. This was also used for the purification of Tr-Cel7A.

Anion exchange chromatography using a Q Sepharose column (HiTrapTM 5 ml QHP, GE HealthCare, USA) and phosphate buffered saline (PBS) at pH 5.5 was used for purification. A linear NaCl gradient from 0 M to 1 M NaCl at 2 ml min\(^{-1}\) flow rate was used for eluting the protein. The purified Tr-Cel7A was used for further analysis.  

**Assay for Tr-Cel7A activity:**

The *p*-nitrophenyl-β-D-cellobioside (pNPC) (Sigma, USA) was used as the substrate to assay the Tr-Cel7A activity [20]. The specific activity and the extracellular activity of Tr-Cel7A were determined by incubating the purified Tr-Cel7A protein and the supernatants collected from cell culture via centrifugation (15 min at 10,000 × g), respectively, following the described method [11]. One unit of Tr-Cel7A activity was defined as the amount of enzyme required to release 1 μmol of pNP from the pNPC in 1 min at 50°C and pH 5.0. The protein concentration of purified Tr-Cel7A was determined using the BCA Protein Assay Kit (Beyotime biotechnology, China). And the dry weight of yeast cells was determined as previously described [11].

**Determination of the Tr-Cel7A expression level:**

The open reading frame (ORF) of Tr-Cel7A was amplified from pYX242MH using the primers for Tr-Cel7A (5'GACTCGAGCATATGGATATCCGTTTTGAG-3' and 5'GCTCGAGTTGGCTGGAATGCTGGTTG-3'), and digested with XhoI and PstI to insert into the pYX242MH. Then the plasmid pYX242MH was transformed into the recombinant strain CEN.PK102-3A derivative; {pJCF}/(Tr-cel7A-FLAG). The resulting transformants were cultured in SC-Ura (1.7 g l\(^{-1}\) yeast nitrogen base, 5 g l\(^{-1}\) ammonium sulfate, and 20 mg l\(^{-1}\) histidine) media supplemented with 200 μg l\(^{-1}\)*p*-nitrophenyl-β-D-cellobioside. The cell-free culture supernatants were collected from cell culture via centrifugation (15 min at 10,000 × g), respectively, following the described method [11].

**Table 1: Strains and plasmids used in this work.**

| Strain and plasmid | Relevant genotype | Source |
|--------------------|------------------|--------|
| *Saccharomyces cerevisiae* | | |
| CEN.PK102-3A | MATa ura3-52 leu2-112 | [18] |
| BSX000 (Control) | CEN.PK102-3A derivative; pJFE3 | [11] |
| cel7AF (Wild type) | CEN.PK102-3A derivative; {pJCF}/(Tr-cel7A-FLAG) | [11] |
| mnn1Δ/cel7AF | CEN.PK102-3A derivative; mnn1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG) | This work |
| mnn9Δ/cel7AF | CEN.PK102-3A derivative; mnn9::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG) | This work |
| och1Δ/cel7AF | CEN.PK102-3A derivative; och1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG) | This work |
| mnn1Δ/mnn9Δ/ | CEN.PK102-3A derivative; mnn1::loxP-KanMX-loxP; mnn9::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG) | This work |
| och1Δ/cel7AF | CEN.PK102-3A derivative; och1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG) | This work |
| MDS1/cel7AF | MDS1/cel7AF derivative; {pYX242MH}/(Tr-MDS1-HA) | This work |
| mnn1Δ/och1Δ/ | mnn1::loxP-KanMX-loxP; och1::loxP-KanMX-loxP; {pJCF}/(Tr-MDS1-HA) | This work |
| MDS1/cel7AF | MDS1/cel7AF derivative; {pYX242MH}/(Tr-MDS1-HA) | This work |
| Plasmids | | |
| pJFE3 | TEF1p-PGK1t | [35] |
| pJCF | pJFE3; TEF1p-Tr-cel7A-FLAG-PGK1t | [11] |
| pAJ401mds1 | Tr-MDS1 | [15] |
| pYX242 | TEF1p-polyA | [36] |
| pYX242MH | TEF1p-Tr-MDS1-HA-polyA | This work |

**Western blot analysis:**

Western blot analysis of total secreted proteins at 48 h was performed using the anti-FLAG monoclonal antibody OctA-Probe (Santa Cruz, USA) as the primary antibody to detect Tr-Cel7A. Horseradish peroxidase-conjugated affinipure goat anti-rabbit IgG antiserum (Zhongshan Goldenbridge Biotechnology Co., Ltd, China) was used as the secondary antibody. The chemiluminescent detection system (Amersham ECL, USA) was used to visualize protein bands. The membranes were stained with Coomassie Blue to check the loading of the protein samples. The cell-free culture supernatants were collected from cell culture via centrifugation (15 min at 10,000 × g). The specific activity of purified Tr-Cel7A was determined using the BCA Protein Assay Kit (Beyotime biotechnology, China). And the dry weight of yeast cells was determined as previously described [11].

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was used as the secondary antibody. The immune-reactive proteins were visualized using ECL (Thermo Scientific, Pierce, USA) and detected using an ImageQuant 400 imaging system (GE Healthcare, USA).

**Determination of Tr-Cel7A concentration by ELISA system:**

The cells were incubated in 100 mL SC-Ura and SC-Leu-Ura medium. The culture supernatants were concentrated approximately 100-fold using Amicon Ultra-15 centrifugal filter units (Millipore, USA) equipped with 30 kDa cutoff membranes. Tr-Cel7A quantitation was performed following the procedure described previously [11] using the ELISA method [21].

**Statistical analysis of data:**

A two-tailed t-test, assuming equal variances, was used to determine whether the differences were statistically significant.

**Results**

The deletion of the endogenous mannosyltransferases decreased the glycosylation of Tr-Cel7A:

The secreted Tr-Cel7A from *S. cerevisiae* was proved to be N-hyperglassylated [9-11]. Our previous result showed that the N-glycosidase F (PNGase F) removed almost all types of the N-linked hyperglycosylation [9-11]. Our previous result showed that the N-glycosylation of recombinant Tr-Cel7A protein secreted by *S. cerevisiae* and lessened the molecular masses from over 100 kDa to two small types, which were ~90 and ~45 kDa [11]. To decrease the elongation of the outer chain of the N-linked oligosaccharides, the yeast α-1,6-mannosyltransferases, Och1p and Mnn9p, and the α-1,3-mannosyltransferase, Mnn1p, which were involved in the N-glycosylation process, were disrupted singly or doubly. As expected, all the single deletion mnn1Δ, mnn9Δ and och1Δ increased extracellular Tr-Cel7A activities (Figure 2a). The double deletion showed an additive effect on its extracellular activities (Figure 2b). At 48 h, mnn1Δ/och1Δ increased its extracellular activity by 272 ±2%, compared with the wild type strain cel7AF. Western blot analysis showed that all these modifications decreased the molecular masses of Tr-Cel7A. The absence of the Och1p or Mnn9p reduced the molecular masses of Tr-Cel7A more significantly and showed two bands at about 75 kDa and 45 kDa both in single- and double-modified strains (Figure 2c). This indicated that glycosylation of Tr-Cel7A decreased by deleting the endogenous mannosyltransferases thus improved the extracellular activity. Meanwhile the specific activity with purified proteins by the Q Sepharose column and the secretion yield of Tr-Cel7A from mnn1Δ/och1Δ were determined respectively. The specific activity of Tr-Cel7A proteins from mnn1Δ/och1Δ was increased by 175 ± 1%, and its secretion yield detected by ELISA assay was also increased by 86 ± 6%, compared with that of the wild type strain cel7AF (Figure 3a and 3b, column 1 and 2). This indicated that reduction in the glycosylation on Tr-Cel7A improved not only the specific activity but also the secretion yield.

The expression of α-1,2-mannosidase Tr-Mds1p in *S. cerevisiae* ulteriorly decreased the molecular weight of Tr-Cel7A: The core structure (Man3GlcNAc2) of modified glycoprotein in endoplasmic reticulum is same in both *T. reesei* and *S. cerevisiae*. In the following secretion process, only one of α-1,2 mannosyl linkages was digested in *S. cerevisiae* [22], but in *T. reesei* all three α-1,2 mannosyl linkages were cleaved by Tr-Mds1p inside and outside of the cells [15,17]. Therefore, we expressed the Tr-Mds1p with its native signal peptide in *S. cerevisiae* strains. The molecular masses of recombinant Tr-Cel7A became visibly smaller (Figure 4a, Line 1 and 2) with extracellular activity improved by 26 ± 1% at 48 h (Figure 4b) in the wild type background. When Tr-Mds1p was expressed in the mnn1Δ/och1Δ strain, more Tr-Cel7A proteins gathered at the status around 75 and 45 kDa (Figure 4a, Line 4), and the extracellular activity of Tr-Cel7A was further improved to 66.5 ± 2.1 mU g dry weight of cells (P<0.01) at 48 h, which was 320 ± 8% higher than that in wild type strain (Figure 4b). This indicated that expression of α-1,2-mannosidase Tr-Mds1p in *S. cerevisiae* ulteriorly decreased the glycosylation of Tr-Cel7A, and thereby affected its extracellular activity. Meanwhile, about 45 ± 8% and 16 ± 3% improvements in the specific activity and the secretion yield of Tr-Cel7A were obtained when Tr-Mds1p was expressed in mnn1Δ/och1Δ (Figure 3b and 3c).
column 3). As it in T. reesei [12], the recombinant Tr-Mds1p was detected both inside and outside cells of S. cerevisiae by western blot analysis (data not shown). This revealed that T. reesei source α-1,2-mannosidase Tr-Mds1p reduced the glycosylation extent of the Tr-Cel7A in S. cerevisiae. Such glycosylation state change improved not only the specific activity but also the secretion yield.

**Figure 3:** Deletion of yeast mannosyltransferases increased the specific activity and secretion yield of Tr-Cel7A. (a) The purified Tr-Cel7A protein by the Q Sepharose column was used for tested its specific activity with pNPC as the substrate. (b) The secretion yield was detected by ELISA assay with anti-Flag antibody. The samples were secreted protein precipitated from culture supernatants at 48 h. All data shown are the mean values (± standard error) obtained from three independent experiments. The differences between the modified strains and wild type were all significant P<0.01 (**).

**Discussion**

Hyperglycosylation of S. cerevisiae inhibited the activity of recombinant Tr-Cel7A enzyme, a glycoprotein from T. reesei, significantly [11,23]. In order to investigate if the glycosylation in S. cerevisiae close somewhat to its native state could bring benefit to the extracellular Tr-Cel7A activity, the protein glycosylation engineering including disruption of the yeast mannosyltransferases Och1p, Mnn9p and Mnn1p, and heterogeneous expression of T. reesei mannosidase Tr-Mds1p, was performed. The speculative structures of N-glycans on Tr-Cel7A proteins after modifications were compared with that of the wild type strain in Figure 5. All modifications increased extracellular activities of Tr-Cel7A and decreased its glycosylation. Based on mnn1Δ/och1Δ, expression of Tr-Mds1p enhanced the extracellular Tr-Cel7A activity up to 320 ± 8% higher than the wild type strain (Figure 4b). The glycosylation reduction improved not only the specific activity but also the secretion yield of the Tr-Cel7A.

**Figure 4:** The heterogenous α-1,2-mannosidase Tr-Mds1p decreased the glycosylation (a) and increased the extracellular activity (b) of Tr-Cel7A. (a) The secreted proteins were precipitated from culture supernatants at 48 h. The Tr-Cel7A proteins were detect by western blot with anti-Flag monoclonal antibody. (b) The extracellular Tr-Cel7A activity was assayed with culture supernatants using pNPC as the substrate. The definitions of the “Control” and “Wild type” were shown in Table 1. All data shown are the mean values (± standard error) obtained from three independent experiments. The differences between the modified strains and wild type were all significant (P<0.05) even more significant (P<0.01) at 48h.

**Figure 5:** The speculative structures of N-glycans on Tr-Cel7A proteins in wild type and modified S. cerevisiae strains

The molecular mass of core Tr-Cel7A deduced from amino acid residues with no glycosylation is about 46 kDa. However, because of the changes in the glycosylation extent, Tr-Cel7A native status with different molecular masses (from ~57 to ~70 kDa) were detected in T. reesei [12]. Same as in Penicillium decumbens, the native Pd-Cel7A also showed different molecular masses (from ~57 to ~74 kDa) [24]. For same reason, in S. cerevisiae, the secreted Tr-Cel7A with the same amino acid residues sequence also showed different molecular masses even over 100 kDa, which meant it was hypglycosylated. However, protein secretory pathway reconstruction including glycosylation engineering can significantly decrease the molecular mass of Tr-Cel7A produced by S. cerevisiae, declining the glycosylation degree close to its core molecular weight [11] (Figure 2c and Figure 4a). Such
though, the extracellular activity produced by S. cerevisiae is still quite 
in the N-deglycosylation, affected the glycosylation of Tr-Cel7A in S. 
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function without Och1p. Therefore, the double deletion of och1 and 
and made more Tr-Cel7A proteins gather at the status around 75 kDa 
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