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Protein disulfide isomerase homolog TrPDI2 contributing to cellobiohydrolase production in *Trichoderma reesei*

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ABSTRACT

The majority of the cysteine residues in the secreted proteins form disulfide bonds via protein disulfide isomerase (PDI)-mediated catalysis, stabilizing the enzyme activity. The role of PDI in cellulase production is speculative, as well as the possibility of PDI as a target for improving enzyme production efficiency of *Trichoderma reesei*, a widely used producer of enzyme for the production of lignocellulose-based biofuels and biochemicals. Here, we report that a PDI homolog, TrPDI2 in *T. reesei* exhibited a 36.94% and an 11.81% similarity to *Aspergillus niger* TIGA and *T. reesei* PDI1, respectively. The capability of TrPDI2 to recover the activity of reduced and denatured RNase by promoting refolding verified its protein disulfide isomerase activity. The overexpression of *Trpdi2* increased the secretion and the activity of CBH1 at the early stage of cellulase induction. In addition, both the expression level and redox state of TrPDI2 responded to cellulase induction in *T. reesei*, providing sustainable oxidative power to ensure cellobiohydrolase maturation and production. The results suggest that TrPDI2 may contribute to cellobiohydrolase secretion by enhancing the capability of disulfide bond formation, which is essential for protein folding and maturation.

1. Introduction

The lignocellulose-based production of biofuels and biochemicals requires a saccharification process in which the cellulose and hemicellulose are degraded to fermentable sugars by the corresponding cellulases and hemicellulases [1]. The high cost of lignocellulolytic enzymes still hinders the commercialization of lignocellulosic biofuels and chemicals [2].

Currently, the industrial production of cellulase is primarily accomplished using the Sordariomycete *Trichoderma reesei* (*T. reesei,* teleomorph *Hypocrea jecorina*), a filamentous fungus. While *T. reesei* grows on cellulosic substances, it secretes extracellular mixtures of cellulases that contain cellobiohydrolase (exoglucanase), endo-β-1,4-glucanase, and β-1,4-glucosidase, which synergistically degrade cellulose to oligosaccharides and glucose with the help of polysaccharide monooxygenases' (PMOs, CDHs, CBM33) oxidative cleavage of cellulose [3]. Throughout the cellulase production, nascent polypeptides are subjected to protein folding and modification in the endoplasmic reticulum (ER) after translation, before vesicle-mediated transport and secretion [4]. The majority of cysteine residues in extracellular proteins form disulfide bonds that stabilize the protein structure [5]. On the other hand, the formation of disulfide bonds, that requires chaperones and protein disulfide isomerase (PDI)-mediated catalysis, is considered as a rate-limiting step of the folding process of proteins [6].

Eukaryotic PDIs contain N- and C-terminal CGHC motifs and possess four Trx-fold domains [6]. Oxidized PDI catalyzes the formation of disulfide bonds via electron transfer from the reduced protein to the PDI CGHC motif. The reduced cysteine residues of PDI are subsequently reoxidized by another ER-associated thiol oxidase, Ero1, enabling continuous PDI-mediated catalysis [6,7]. The *Saccharomyces cerevisiae PDI1* gene encodes a PDI, which introduces disulfide bond into proteins (oxidase activity) and provides quality control by catalyzing the rearrangement of incorrect disulfides (isomerase activity) *in vitro* [8]. This gene has been proven to be essential for yeast growth [8,9]. The oxidase function of Pdi1p was demonstrated to be of greater importance and was sufficient to maintain yeast growth, even when there was a lack of isomerase

Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonicacid; DTT, dithiothreitol; ER, endoplasmic reticulum; MM, minimal medium; PDI, protein disulfide isomerase; rdCBH1, reduced and denatured CBH1; rdRNase, reduced and denatured RNase; ., *TT reeseiTrichoderma reesei*.

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Table 1
Primers used in this study.

Primer	Sequence
TrPDI2-1F	5′-AAGGTACCATGGTCTTGATCAAGAGCC-3′
TrPDI2-1R	5′-AAGGATCCTCACAGCTCGTCCTTCTGG-3′
TrPDI2-2F	5′-AATCTAGAATGGTCTTGATCAAGAGCC-3′
TrPDI2-2R	5′-AAATGCATTCACAGCTCGTCCTTCTGG-3′
TrPDI2-3F	5′-AAGGATCCATGGTCTTGATCAAGAGCC-3′
TrPDI2-3R	5′-AAGAATTCTCACAGCTCGTCCTTCTGG-3′
Ppkisc-F	5′-GTGAGACTAGCGGCCGGTCCCTT-3′
TrPDI2sc-R	5′-TCACAGCTCGTCCTTCTGGTCCT-3′
TrPDI2KO-5F	5′-GTAACGCCAGGGTTTTCCCAGTCACGACGGTAGCATTCAACGTGACAGG-3′
TrPDI2KO-5R	5′-CGACGATATCAGCTTCCATATTCCGACTAGAAGATAAGCGCATCACAGC-3′
TrPDI2KO-3F	5′-AGAAAAGCACAAAGAAGAGGCTCCAACTAAGAACTATACGACGAGGAGG-3′
TrPDI2KO-3R	5′-GCGGATAACAATTTCACACAGGAAACAGCAACATTCCACTGAGGTGAGG-3′
pyr4-F	5′-*TAGTCGGAATATGGAAGCTGATATCGTC*GGCCTCTTCTTTGTGCTTTTCT-3′
pyr4-R	5′-*TAGTTGGAGCCTCTTCTTTGTGCTTTTCT*ATATGGAAGCTGATATCGTCG-3′
TrPDI2sc	5′-ATTGTACGTTAGATCAAGTAG-3′
pyr4sc	5′-CCTCTTTTTCCATCTTGTC-3′
pdi1-F	5′-CGTTGTCGTTGCCCACT-3′
pdi1-R	5′-TCGCTCTTGGCATACAGG-3′
pdi2-F	5′-CCCACCATCCTCTTCTTCC-3′
pdi2-R	5′-CTCCGCAGCATCCGTAA-3′
tef1-F	5′-GCTCTGCTCGCCTACACCCT-3′
tef1-R	5′-TCTCCTTCTCCCAGCCCTTG-3′

activity [9]. Previously, the *T. reesei pdi1* gene was characterized as functional complement to the corresponding yeast mutant [10]. Its expression increased following dithiothreitol (DTT) treatment and during heterologous protein production [10]. However, the role and mechanism of PDI in cellulase production and the possibility of PDI being a factor in the improvement of the enzyme production efficiency of *T. reesei* remain speculative.

Here, we report a PDI-encoding gene, *Trpdi2*, evaluate its contribution to cellulase production and thoroughly analyze the underlying mechanism. TrPDI2 manifested enzymatic activity to reduced and denatured RNase A and cellobiohydrolase (CBH1) *in vitro*, and *in vivo* contributed to cellobiohydrolase production. In addition, TrPDI2 was able to catalyze the formation of disulfide bonds and thus recover the activity of reduced and denatured CBH1. Moreover, the expression level and redox state of TrPDI2 respond cellulase induction to guarantee sustainable oxidative power in *T. reesei*. As a result the increased expression of *Trpdi2* may promote cellobiohydrolase maturation and secretion.

2. Materials and methods

2.1. Phylogenetic analysis and sequence alignment

Protein sequences were obtained from the Joint Genome Institute *T. reesei* (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and Aspergillus genome databases (http://www.aspergillusgenome.org/), and NCBI (http://www.ncbi.nlm.nih.gov/guide/proteins/). The ClustalW and MEGA5 programs were used to construct a phylogenetic tree of proteins with CXXC catalytic motifs, using the minimum evolution method and 500 bootstrap replications as a test of phylogeny. DNAMAN (version 6.0) was used for the alignment of the amino acid sequences.

2.2. Strains and culture conditions

Escherichia coli strains DH5α and BL21 (DE3) were used as the host strains for recombinant plasmid construction and protein production, respectively. The yeast strain Δ*Scpdi1* (*MAT* a/α *his3*Δ/*his3*Δ *leu2*Δ/*leu2*Δ *MET15*/*met15*Δ *LYS2*/*lys2*Δ *ura3*Δ/*ura3*Δ *pdi1*Δ/*PDI1*, EUROSCRAF) was used for the *Trpdi2* complementation experiment. *T. reesei* strain QM9414 (ATCC 26921), a uridine auxotrophic strain TU-6 (ATCC MYA256) and a strain defective in the nonhomologous end-joining pathway, Δ*tku70* [11] were used in this study.

Potato dextrose agar media (with 10 mM uridine for TU-6 and Δ*tku70*) was used for the normal growth and maintenance of all *T. reesei* strains. Solid minimal medium (MM) [12] was used to analyze the phenotype of *T. reesei* strains with varying *Trpdi2* expression levels.

For cellulase induction, 10^7 fresh spores were inoculated into a 250 ml Erlenmeyer flask containing 50 ml MM with 10 g/l glucose as carbon source. After a 48 h preculture, the mycelium was filtered onto sterile gauze, washed three times with MM (no carbon source) and then inoculated into 50 ml MM with 3% Avicel as carbon source. For phenotype assessments, fresh blocks of fungus of same size were inoculated onto solid MM plates with glucose as the carbon source.

2.3. Construction of T. reesei strains expressing Trpdi2

The gene segment of *Trpdi2* coding region was obtained with TrPDI2-2F and TrPDI2-2R as primers (Table 1) and *T. reesei* cDNA as template. The *Trpdi2* segment was then separately digested with XbaI and NsiI and ligated into digested pRLMex30 [13], a widely used expression plasmid in *T. reesei*. After confirmation by sequencing, the resulting plasmid (Ppki::*Trpdi2*) was recombined into the genome of TU-6 by co-transformation with pyr4-pBluescript (plasmid harboring gene encoding orotidine-5′-phosphate decarboxylase) as described previously [14]. Strains harboring Ppki::*Trpdi2* were screened by genomic PCR with Ppkisc-F and TrPDI2sc-R (Table 1); the selected strains were subjected to real-time RT-PCR for another confirmation at transcriptional level after spore separation. Three confirmed strains with different expression levels were used for further analysis.

2.4. Protein production and purification

The *Trpdi2* PCR products from PCR reactions with Trpdi2-1F and Trpdi2-1R used as primers (Table 1) were digested with NcoI and BamHI, purified and subcloned into the expression vector pET24d, which comprises the coding sequence for a hexahistidyl affinity tag at the N terminus of target protein.