A novel Swollenin from *Talaromyces leycettanus* JCM12802 enhances cellulase hydrolysis towards various substrates

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Research

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Abstract

Background: Swollenins exist within some fungal species such as *Trichoderma reesei* and *Aspergillus niger*, and are candidate accessory enzymes for the biodegradation of cellulosic substrates. Swollenins theorized role in biological processes is as promoters in the rearrangement of non-covalent interaction networks formed by cell wall polysaccharides. Such network rearrangements though to be responsible for expanding accessibility for hydrolytic enzyme degradation processes. We hereby report in detail the characterization of a recombinant swollenin and its disruptive activity on cellulosic substrates and its synergistic effects while interacting with cellulases.

Results: We have expressed a novel swollenin gene *Tlsw* consists of an open reading frame encoding 503 amino acids to be identified from *Talaromyces leycettanus* JCM12802 and successfully expressed it in both *Trichoderma reesei* and *Pichia pastoris*. Similar to other fungal swollenins, *TlSWO* is composed of a N-terminal family 1 carbohydrate binding module (CBM1) followed by a Ser/Thr rich linker connected to an expansin-like domain, followed by a family of 45 endoglucanase-like domains and a group-2 grass pollen allergen domain. From our experimental results, *TlSWO* demonstrated disruptive activity on Avicel and displayed a synergistic effect with cellobiohydrolases, enhancing its hydrolytic performance up to 132%. We also explored the activity of *TlSWO* on several model substrates as well as pretreated biomass. Our results indicate that *TlSWO* is capable of releasing reducing sugars from lichenan, barley β -glucan, carboxymethyl cellulose sodium (CMC-Na) and laminarin. The specific activity of *TlSWO* towards the substates mentioned above is 9.0 ± 0.100 U/mg, 8.9 ± 0.100 U/mg, 2.3 ± 0.002 U/mg and 0.79 ± 0.002 U/mg, respectively. Moreover, further experimentation confirms the fungal swollenin *TlSWO* exhibits maximum activity at pH 4.0 and 50 °C.

Conclusion: This study reports on a novel swollenin protein capable of enhancing biomass conversion. Experimental data reveals the functional diversity of this swollenin with considerable activity on various substrates. Although the exact catalytic mechanism of swollenin's remains unknown, the functional diversity of *TlSWO* broadens its applicability under experimental settings, and suggests it may be a promising candidate for future industrial applications.

Background

Lignocellulosic biomass has been considered as an alternative to fossil fuels, however challenges still remain for biomass feedstocks such as this to be properly processed and converted into fuels or other chemical production purposes [1]. A successful pretreatment process is measured two ways. First, by the highly efficient recovery of carbohydrates from within the raw materials. And second, by minimizing the formation of toxic and inhibiting compounds to prevent unwanted health risks and environmental hazards [2]. The enzymatic deconstruction of biomass that follows pretreatment can be enhanced by using a number of non-glycoside hydrolase accessory enzymes. These enzymes include but not limited to expansins, loosenins, ceratoplatanin proteins, and certain other types of carbohydrate binding modules

[3-6]. Among these proteins, expansins have been widely found in plant cell walls and studies have shown that they are responsible for loosening the cellular wall [7].

Studies done by McQueen-Mason have proposed that expansins have the ability to disrupt hydrogen bonding between plant cell wall polysaccharides without hydrolyzing them first [8]. In 2002, the first expansin-like protein SWOI from fungi was discovered in *Trichoderma reesei* [9] and because swollenins from fungi in many studies are shown to have sequence similarity to expansins, they are now often referred to as expansin-like proteins. This study also concluded that the SWOI protein was able to disrupt cotton fibers and filter paper structures on a microscopic level without detectable reducing sugars [9]. Over the past decade, scientists have identified more than 10 types of swollenins [10-13]. And recently, a new database, Expansin engineering Database (ExED, <https://exed.biocatnet.de>), has been released to the public [14]. In general, expansins have a length of no more than 250 amino acids which constitute a two-domain structure. The primary domain resembles the glycoside hydrolase family 45 (GH45) and such homology preserves certain sequence features of the GH45 catalytic site [15]. The second domain has a characteristic flat aromatic-rich surface and is homologous to group-2 grass pollen allergens. In some studies, this domain was proposed to function as a CBM [16, 17]. The two domains within the expansin are interconnected by a short linker, and both domains are required for plant cell-wall loosening activity.

However, there are functional differences from structural discrepancies between swollenins and expansins. For example, swollenins have an additional CBM domain, allowing a display of homology to fungal cellulases in the N-terminal [18]. In other cellulases, CBMs direct the enzymes to bind on cellulosic surfaces and thus enhance lignocellulose degradation [19-21]. Also, *O*-glycosylation of linkers may also have some effect on the binding of the enzyme to surfaces [22].

Previous studies have also described the synergy of swollenin with glycoside hydrolases in releasing soluble sugars from substrates. As a prominent example, Zhou *et al.* were able to successfully express swollenin SWO2 in *Aspergillus niger*, showing that the simultaneous incubation of SWO2 with cellulases resulted in a significant increase in synergistic activity in cellulose hydrolysis. This synergistic activity was improved even further when the cellulose was pretreated with swollenin first [12]. Other investigations focused on the synergy of swollenin with xylanase by Santos *et al.* found that a swollenin ThSWO from *T. harzianum* created a rough and amorphous surface on Avicel and also displayed a high synergistic effect with a commercial xylanase from *T. viride*, enhancing its hydrolytic performance up to 147% [11]. Furthermore, Anthony *et al.* indicated in their study that a chimeric enzyme with swollenin from *T. reesei* fused with feruloyl esterase A from *A. niger* was also found to produce a significant increase in ferulic acid released from lignocellulose samples [23]. Other studies demonstrated that swollenins also have the capability to release reducing sugars from cellulosic materials. For example, swollenin SWO2 from *T. pseudokoningii* and AfSwo1 from *Aspergillus fumigatus* were both found to exhibit very low level of endoglucanase activity [12, 24]. Recently, Andberg *et al.* demonstrated swollenin SWOI from *T. reesei* expressed activity on substrates containing β -1,4 glucosidic bonds, hypothesized a unique mode of mechanistic action with similarities in both endoglucanases and cellobiohydrolases [25].

In our previous work, we identified a thermophilic *T. leycettanus* strain JCM12802 which is an excellent CAZyme source [26-29]. In this study, we present a novel swollenin protein *T*/SWO that was cloned from *T. leycettanus* JCM12802, and successfully expressed in *T. reesei* and *Pichia pastoris*. We have examined *T*/SWO activity performance on various substrates, demonstrating *T*/SWO's capability in releasing reducing sugar from barley β -glucan, lichenan, laminarin, and carboxymethyl cellulose sodium (CMC-Na). In addition, we show that *T*/SWO was able reduce the particle size and the surface structure of Avicel while significantly increase synergistic activity by up to 72.2% on pre-treated corn stover (PCS).

Results

Identification and characterization of *T*sw gene

The ORF of putative *T*sw consists of six exons, encoding a protein (*T*/SWO) of 503 amino acids and a signal peptide at the cleavage site between amino acids 20 and 21. The cloning sequence of putative *T*/SWO was submitted to NCBI GenBank as MT180127. The predicted product shows the highest sequence similarity to the amino acid sequence of the known swollenin which are from *Aspergillus fumigatus* or *Penicillium oxalicum*. Further analysis by PROSITE demonstrated that *T*/SWO consists of three domains, fungal-type carbohydrate-binding module family 1 (CBM1) (amino acids 23-59), family 45 endoglucanase-like domain of expansin (Expansin_EG45) (amino acids 206-388) and a cellulose-binding-like domain of expansin (Expansin_CBD) (amino acids 400-492), which are typical of the swollenins from fungi (Fig. 1). In the CBM1 of *T*/SWO, six cysteines were highly conserved. Disulfide bond prediction by DiANNA showed that there are three disulfide bonds in the CBM1 of *T*/SWO (Cys4-Cys21, Cys11-Cys28, Cys22-Cys28). CBM1 and Expansin_EG45 are connected by a Serine-Threonine rich linker domain. Although the function of linker has been studied in other cellulases, it is not clear if the linker plays any similar role in swollenins. Sequence alignment of swollenins showed *T*/SWO maintained a conserved HMD (histidine, methionine, aspartic acid) catalytic motif of the GH45 cellulase (HFD, histidine, phenylalanine, aspartic acid), which is part of the active site (Figure 1). In the GH45 HFD, the aspartic acid is the proton donor during the catalytic process. However, the other residue of the catalytic active site, aspartic acid, is absent from both swollenin and part of GH45 cellulases. In the C-terminal region of *T*/SWO, the expansin_CBD is homologous to pollen allergen. There are also several conserved aromatic amino acids in the sequence, i.e., Y400, Y401, F402, W429, Y447, W450, Y496 and F503, which may play a key role in binding substrates (Figure 1).

Expression of *T*/SWO in *P. pastoris* GS115 and *T. reesei* AST1116

Expression of *T*/SWO was preformed using the *aox1* promoter and *eno* promoter in *P. pastoris* and *T. reesei* respectively. Following high-throughput fungal expression and screening, a large set of colonies resulting in SDS bands after transformation were identified. The results revealed that recombinant *T*/SWO (483 amino acid residues, 51.1 kDa and a theoretical isoelectric point of 4.45) was successfully expressed using *P. pastoris* and *T. reesei* (Figure S1). The purified swollenin protein migrated as an about ~80 kDa protein in SDS-PAGE. Due to the difference between the SDS-band and the theoretical molecular

weight, a single band was analyzed by MALDI-TOF MS and the trypsin-digested peptide sequences were matched to the deduced amino acid sequences of *T/SWO* (Figure S2). Sequence prediction suggested that there are five N-glycan sites on *T/SWO* (Asn35, Asn154, Asn249, Asn366 and Asn436). After Endo H digestion, *T/SWO*'s molecular weight decreased to ~72 kDa which is still higher than theoretical MW (Figure S1A). We speculate that the rest of the molecular weight increase was caused by heavy *O*-glycan glycosylation in the linker region which is rich in serines and threonines and reported for other proteins expressed in these hosts.

Activity of *T/SWO* on different substrates

T/SWO's cellulolytic activity was measured with lichenan, barley β -glucan, CMC-Na, laminarin, avicel, glucomannan, the xylanase activity was measured with birchwood xylan, and the mannase activity was measured with locust bean gum. All reactions were carried out overnight. Our results show that *T/SWO* only has significant activity on lichenan, barley β -glucan, glucomannan, and CMC-Na, and a very low activity on laminarin. *T/SWO* showed the highest activity on lichenan (9.0 ± 0.100 U/mg) and barley β -glucan (8.9 ± 0.100 U/mg), followed by CMC-Na (2.3 ± 0.002 U/mg). In contrast, only slight activity was observed against laminarin (0.79 ± 0.002 U/mg) (Figure 2). These findings suggest that *T/SWO* mainly acts on cellulose rich substrates and shows a preference to the substrates that contain 1,4 linkages.

Mode of action of *T/SWO*

The *T/SWO*'s mode of action was assessed using the aforementioned four substrates (Figure 3). As a result, CMC-Na was hydrolyzed into cellobiose and small amount of cellotriose (Figure 3). Analysis of the hydrolysis products of lichenan, barley β -glucan showed that *T/SWO* preferentially hydrolyzed these two substrates into products with different degrees of polymerization, including cellobiose and cellopentose, followed by cellohexose and cellotetrose (Figure 3). We detected no sugar release after incubating *T/SWO* with laminarin (Figure 3). These results suggested that *T/SWO* may have a function of endo-cellulase.

Effect of temperature and pH on *T/SWO*

The effect of pH and temperature on *T/SWO* activity were further investigated with lichenan as a substrate. We determined the optimum pH is 4.0 while the enzyme displayed activity across a broad pH range of 2.0–12.0 (Figure 4A). As for pH stability, *T/SWO* retained more than 80% of its activity within the pH range 2.0–9.0 after incubation at 37 °C for 1 h. In contrast, *T/SWO* lost 30% of its activity after incubation at pH 10.0–12.0 after 1 h (Figure 4B). In addition, *T/SWO* reached optimal activity at 50 °C and retained more than 90% of its activity within the temperature range of 40–60 °C. However, after heating to 70 °C or above, its activity falls off very rapidly (Figure 4C). Also, as shown in figure 4D, *T/SWO* maintained stable activity between 37 °C and 50 °C after 1 hour of incubation, however, its activity quickly decreased to 40% after a 10 minute incubation as temperature was raised to 70 °C.

Disruptive action of *T/SWO* on Avicel

The disruptive effect of *T/SWO* on Avicel was evaluated using light microscopy (LM) and scanning electron microscopy (SEM). LM analysis showed that after incubation with varied amounts of *T/SWO* for 24 h, the Avicel's physical structure tend to be significantly different from the untreated Avicel (Figure 5). As we increased the amount of *T/SWO*, Avicel was disrupted into smaller particles. The sample that was pretreated with 300 mg of *T/SWO* for 12 h was taken out for further analysis using SEM. As we can see, *T/SWO* created a rough and amorphous surface on Avicel compared with the untreated sample (Figure S3).

Synergism between *T/SWO* and cellulases

To test the capacity of *T/SWO* to enhance biomass hydrolysis by enzymatic cocktail, we hydrolyzed pretreated biomass using cellulases alone first, followed by treatment using both cellulases and *T/SWO*. Biomass degradation experiments were performed using β -glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91) and endoglucanase (EC 3.2.1.4) in the presence of *T/SWO*. The reactions with BSA and without *T/SWO* were used as controls. The total protein used was 13 mg protein/g of glucan in all reactions. Since endoglucanases randomly cleave the internal β -1,4-glycosidic bonds creating new reducing ends, this allows cellobiohydrolases to continuously act on the chain termini to release cellobiose and β -glucosidase that hydrolyzes cellobiose into glucose [30], therefore only the production of glucose was compared in different reactions.

Our results showed that *T/SWO* exhibited significant synergetic effects on cellobiohydrolase Cel7A when using PCS as the substrate. Initially, we measured the reaction's glucose quantity containing only 13 (mg protein/g of glucan) of *T/SWO*. No glucose was detected in the reaction, suggesting that PCS could not be hydrolyzed to monomers by *T/SWO* alone (Figure 6). When the reaction contained Cel7A and β -glucosidase individually PCS conversion achieved an increase from 8.9% to 16.4% within the 24 h to 120 h period. When Cel7A and β -glucosidase were combined with 2 (mg protein/g of glucan) *T/SWO* the increase changed from 11.2% at 24 h to 26.4% at 120 h. Even though *T/SWO* action alone did not lead to any detectable levels of released glucose, the enzyme addition to Cel7A and β -glucosidase led to a significantly enhanced hydrolytic activity of the cocktail on PCS.

PASC and CNC exist in amorphous and crystalline forms, respectively, which may affect their binding with *T/SWO*. Therefore, the action of *T/SWO* for the two substrates were further examined. Similar to the results of PCS, *T/SWO* could not release any sugars from PASC and CNC when used individually (Figure 7). When utilizing Cel7A and β -glucosidase the conversion results showed that PASC conversion rate reached from 9.9% at 24 h, to 40.2% at 120 h (Figure 7A). However, when Cel7A and β -glucosidase were supplemented by *T/SWO* the conversion rates increased to from 33.0% at 24 h to 72.2% at 12 h. Looking at the 120-hour data, the conversion rate of PASC increased by approximately 32% compared to Cel7A and β -glucosidase individually, suggesting that *T/SWO* exhibited significant synergetic effects with Cel7A. In addition, the synergetic effects of *T/SWO* and endoglucanases were also explored during this research. When using endoglucanase and β -glucosidase individually the conversion rate increase was from 51.4% at 24 h to 85.6% at 120 h. When used in combination with *T/SWO*, the conversion rate reached into from

58.6% at 24 h to 85.7% at 120 h. Although these conversion rates are slightly higher than that of using endoglucanase and β -glucosidase individually, the conversion rate at 120 h was 86.4% is within the margin of error when compared to 85.6%. Therefore, we conclude that the addition of *T*/SWO did not provide a significant increase in enzymatic hydrolysis of PASC when used in combination with endoglucanase.

The cellulose conversion rate of CNC was overall significantly lower than that of PASC. Our results showed that on CNC, the Cel7A and β -glucosidase achieved a conversion rate from 28.2% at 24 h to 59.3% at 120 h (Figure 7B), which was increased further when *T*/SWO was added. The glucose yields obtained by the cocktail enzyme systems containing *T*/SWO and Cel7A and β -glucosidase were 31.9% at 24 h to 68.9% at 120 h, higher than all other cases when compared with the control group. Therefore, we conclude that *T*/SWO showed a synergistic effect with processive cellobiohydrolases. However, when used in combination with endoglucanases, *T*/SWO did not present any significant synergistic effect. After 120 h of enzymatic hydrolysis, glucose yield released from CNC was 16.4% with combined use of *T*/SWO, endoglucanase and β -glucosidase, which corresponds well with the hydrolysis rate of 14.9% using endoglucanase and β -glucosidase individually. Comparing with our PASC results, we hypothesize *T*/SWO will also act on amorphous cellulose more efficiently than their respective crystalline forms.

Discussion

Several previous studies initially showed the disruptive capabilities of SWOI on plant cell wall structures without leaving any traceable amount of reducing sugars. However, subsequent research confirmed that SWOI does exhibit some hydrolytic activity on cellulosic substrates with features of both endoglucanases and cellobiohydrolases [9, 25]. Indeed, similarly to SWOI, the other two swollenins, *Af*SWO1 from *A. fumigatus*, SWO2 from *T. pseudokoningii* have also showed hydrolytic activity on various substrates, thereby suggesting that these proteins interact with cellulose or hemicellulose [12, 24]. In this study, *T*/SWO from *T. leycettanus* JCM12802 was found to have similar functionalities as other fungal swollenins while sharing a relatively high sequence identity with SWOI, *Af*SWO1 and SWO2 (64.5%, 73.7% and 63.2%, respectively). Although *T*/SWO has the highest activity on lichenan and barley β -glucan substrates, both contain β -1,4 and β -1,3 linkages, its activity becomes very minimal on laminarin, a substrate that only contains β -1,3 linkages. Indicating activity on β -1,4 linkages is the primary mechanism of action. According to previously reports, expansins have higher similarity with GH45 subfamily C enzymes than with other members of the GH45 family [31, 32]. Studies indicating expansins with higher similarity with GH45 subfamily C enzymes than others have a motif termed HFD as part of its active site, which is also present in *T*/SWO (HMD). The aspartic acid in this motif plays the role of proton donor in GH45. Nevertheless, other key residues that are critical for catalytic activities are absent in both expansins and swollenins. This evidence suggests expansins and swollenins may have adopted an inverted mechanism during the catalytic process. In 2015, Nakamura et al. proposed that *Pc*Cel45A which belongs to the GH45 subfamily C, uses an imidic acid form of asparagine residue as a general base in the “Newton’s cradle” proton relay catalytic mechanism [33]. This proposal sheds light on some mechanistic implications of expansin’s catalytic process. Evolutionary process’ horizontal gene transfer (HGT) drives

differences between the fungal swollenins and the plant/bacterial expansins in sequence [34]. These differences could make *PcCel45A*'s catalytic mechanism theory unfeasible when applied to fungal swollenins.

Fungal swollenin sizes are roughly twice as large as plant and bacterial swollenins due to their D1 and D2 domains containing extra sequence insertions such as the additional N-terminal CBM with linkers. CBMs can increase the concentration of their parent enzyme substrate surface, leading to more rapid degradation of polysaccharide [35, 36]. Primary amino acid sequence analysis using BLAST indicates that *T/SWO* contains an N-terminal CBM region (amino acid residues 21–59) that shows the highest similarity towards the fungal GH6 family 1 CBMs. Six typical conserved cysteines which could form three pairs of disulfide bonds. Also three conserved aromatic residues which are typical in GH6 and GH7 cellulases CBMs are presented in the *T/SWO*'s CBM (Trp28, Tyr54, and Tyr55). These sequences' key roles are highlighted in their importance in cellulases stability and activity during the reaction. Moreover, it has been demonstrated that the length of linker is crucial for the activity of cellulases [37]. The linker region of *T/SWO* is over 140 amino acids, longer than that of most of reported swollenins and fungal cellulases [38]. Although CBM and linker have been well studied in cellulases, little is currently known about the actual role of these two regions in swollenins.

We have shown that when treated with *T/SWO*, avicel's smooth surface structure transitions into a rough texture. This is consistent with previous studies suggesting a cell wall modification activity usually expressed by swollenin interference [39, 40]. Previous studies have also shown that swollenins can synergize with other enzymes such as cellulases and xylanases [10, 11, 24]. In this study we explored *T/SWO*'s ability to boost cellulosic substrates hydrolysis by different enzymes. When the cellulose was incubated with *T/SWO* and cellobiohydrolases, a greater increase in glucose yields was observed. However, we observed no significant synergistic effect between *T/SWO* and endoglucanases. These results differ from a previous report, in which swollenin exhibited strong synergistic interaction with endoglucanases [3]. We therefore propose that *T/SWO* has better synergistic activity with cellobiohydrolases. As the classical, C_1 - C_x model hypothesized the process of cellulose degradation (C_1 : non-hydrolytic component, C_x : endo- or exo-acting cellulases). One proposal using this model hypothesizes C_1 can disrupt cellulose by displacing hydrogen bonds in the microfibril, leading to a more available structure for C_x [4, 41]. Eibinger *et al.* and Kang *et al.* speculated swollenins, much like endoglucanases can play the possible role of C_1 enzymes due to their disruptive activity in the enzymatic saccharification of lignocellulosic substrates, although the action mode of various C_1 enzymes needs to be further explored [10, 18, 42]. Our results of PASC and CNC treated with or without *T/SWO* were used to compare the effects of swollenin on different crystallinity materials. We show that when PASC was incubated with *T/SWO* and cellobiohydrolases, the total glucose concentration was increased by 32%, compared to incubating with only cellobiohydrolases. However, when using CNC as the substrate, there is no significant difference between the groups with or without *T/SWO*, suggesting swollenin has some additional ability to bind and disrupt the amorphous cellulose than the crystalline cellulose. This may be due to its endoglucanase like activity as demonstrated on the model substrates we previously used, or

possibly because it is able to bind amorphous substrates more easily than crystalline substrates due to its CBM.

Conclusions

In the present study, we report *T*/SWO, from *T. leycettanus* JCM12802. *T*/SWO as an acidic and mesophilic swollenin, showing activity towards lichenan, barley β -glucan, carboxymethyl cellulose sodium and laminarin. A greater increase in glucose yield was observed when clean cellulose substrates were incubated with *T*/SWO and cellobiohydrolases. Moreover, *T*/SWO exhibited synergetic effects on cellobiohydrolase when using PCS and PASC as substrate. However, there were no observed significant synergistic effect between *T*/SWO and endoglucanases, suggesting that *T*/SWO has better coordination with cellobiohydrolases.

Comparing to chemical pretreatment, biological pretreatment utilizing enzyme has massive research potential given the advantages such as low energy consumption, environmentally friendliness, and lower production cost. Different lignocellulosic biomass needs different types of pretreatments because the structural features of cellulose plays an important role in enzyme hydrolysis rate that affects outcomes such as polymerization degree, cellulose crystallization arrangement, surface area accessibility, particles size, and the existence of hemicelluloses and lignin [2, 43]. We show that *T*/SWO directly caused the alteration of the cellulose structure, consequentially increasing its hydrolysis rate. By optimized preconditioning and molecular design, *T*/SWO could be a promising additive for improving lignocellulosic biomass generation performance observing hydrolysis efficiency.

Methods

Strains and Plasmids

T. leycettanus JCM12802 as the donor strain was purchased from Japan Collection of Microorganisms RIKEN BioResource Center (Tsukuba, Japan). *Escherichia coli* Trans I-T1 (TransGen, Beijing, China) was used for routine gene cloning. *T. reesei* AST1116 and *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) were both used as the host for gene expression. The plasmids of pPIC9 (Invitrogen) and pTrEno were used for driving *Tlswo* gene expression in *P. pastoris* and in *T. reesei*. The plasmid construction of pTrEno was according to the description of Linger et al. [44].

Sequence analysis

The DNA and amino acid sequences of *T*/SWO were analyzed using the BLASTx and BLASTp programs (<http://www.ncbi.nlm.nih.gov/BLAST>), respectively [45]. The introns and exons were predicted using the GENSCAN Web Server (<http://genes.mit.edu/GENSCAN.html>) [46]. SignalP 3.0 was used to predict the signal peptide sequence (<http://www.cbs.dtu.dk/services/SignalP>) [47]. The potential N-glycosylation sites were predicted online (<http://www.cbs.dtu.dk/services/NetNGlyc>). Sequence assembly and estimation of the molecular mass and *pI* of the mature peptide were performed using the Vector NTI Suite

10.0 software (Invitrogen). Protein molecular weight and molar extinction coefficients were estimated at the ExPASy tools page (<http://www.expasy.org/tools>). Multiple sequence alignments were performed with Clustal W program from MEGA software 4.0. PROSITE (<http://br.expasy.org/prosite>) analysis the protein domains and functional sites [48]. DiANNA web server (<http://clavius.bc.edu/~clotelab/DiANNA>) is used to predicting the disulfide bond topology in protein [49].

Gene cloning and recombinant protein expression

T. leycettanus JCM12802 was cultured at 42 °C for 3-5 days in medium containing 5.0 g/L (NH₄)₂SO₄, 1.0 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, 0.2 g/L CaCl₂, 10.0 mg/L FeSO₄·7H₂O, 30.0 g/L wheat bran, 30.0 g/L soybean meal, and 30.0 g/L corncob. Genomic DNA of *T. leycettanus* JCM12802 was extracted with the DNA isolation kit (Tiangen) following the manufacturer instructions and was used as a template for PCR amplification. Total RNA isolation and first strand cDNA synthesis were carried out as previously described [50]. PCR amplification was carried out using Fastpfu DNA polymerase and which purchased from TransGen. The fragment of *T/swo* corresponding to the 21–503 amino acid sequence was amplified with primers P1 (5'- GCTCGTGCTCAGAGCAGCTGTGCAGG-3') and P2 (5'- CTAGATTACCTAGGTAACTGCACC-3'). The amplified PCR product was cloned into pPIC9 and pTrEno vector using Gibson Assembly (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol for cloning and expression. *Escherichia coli* Trans I-T1 (TransGen, Beijing, China) which used for routine gene cloning was grown at 37 °C overnight in Luria-Bertani (LB) medium supplemented with 50 mg/mL of ampicillin (Sigma, St. Louis, MO).

When using *P. pastoris* GS115 as the expression host, the recombinant plasmids of pPIC9-*T/swo* was linearized with *Bgl*II (New England Biolabs, UK) and transformed into the expression host via electroporation. The positive transformants were screened on minimal dextrose medium (MD) at 30 °C for 3 or 4 days until the single colonies appeared and placed into shaking tubes for enzyme production according to the protocol described in the *Pichia* Expression Kit (Invitrogen). The large-scale fermentation was performed as the previously described [30]. The recombinant *P. pastoris* GS115 containing pPIC9-*T/swo* was grown at 30 °C in 400 mL BMGY medium in a 1 L shaking flask for 48 hours. Cells were collected and re-suspended again with 200 mL buffered methanol-complex medium (BMMY) medium with 0.5% (v/v) methanol and cultured at 30 °C for 72 h with shaking (200 rpm). Methanol was added into the medium every 24 hours.

When using *T. reesei* AST1116 as the expression host, the recombinant plasmids of pTrEno-*T/swo* were linearized with *Sbf*I (New England Biolabs, UK) and then transformed into *T. reesei* AST1116 via electroporation. Potato dextrose (PD) plates were used for spore production and PDHX plates (PD plates with hygromycin and TritonX-100 at a final concentration of 100 µg/mL and 0.1% respectively) were used for the screening of potential *T. reesei* which allowed to grow for 2 to 3 days at 30 °C. Growth medium for *T/SWO* expression was Mandels and Andreotti medium with 5% glucose (MAG). Subsequently complete medium lactose (CML) was used for the overexpression of the transformants. The medium protocols of MAG and CML were performed following published protocol [44]. For large-scale fermentation, the

positive transformants spore stocks were streaked on potato dextrose agar plates and allowed to grow 2 to 3 days. Then the spore was extracted from the plate and deposited into 1.0 L of MAG medium in a 2.8 L shake flask. The culture was grown at 28 °C with at 225 RPM for 24 h, after which the entire 1.0 L was transferred to 7.0 L of the same medium in a bioreactor.

Then the culture broth were extracted for further analysis by SDS-PAGE and activity assay. Culture broths were clarified via centrifugation and transferred to microcentrifuge tubes. Broths were diluted 3:1 in 4×LDS sample buffer (Life Technologies Corp., Carlsbad, CA, USA) with 50 µL/mL β-mercaptoethanol as a reducing agent. Samples were incubated at 95 °C for 5 min prior to loading onto NuPAGE SDS gels with MOPS buffer, electrophoresed at 200 V constant for approximately 40 min.

Protein purification

Fermentation broths were harvested and sequentially vacuum-filtered. This filtered broth was then concentrated by tangential ultrafiltration with a 10 kDa MWCO. The broths were roughly concentrated to 100 mL. The final concentrated volume was exchanged with at least 2.0 L of 20 mM Bis-Tris pH 6.5 to remove residual peptides and other low molecular weight debris. The following purification steps were then performed according to previous publications [44]. The crude enzyme was purified through hydrophobic interaction chromatography (HIC) using a 26/10 Phenyl Sepharose Fast Flow column (GE Healthcare, Chicago, USA). Then the protein went through anion exchange chromatography using a 10/100 anion exchange column packed with Source 15Q (GE Healthcare, Chicago, USA) followed by HIC using a Source15 iso 10/100 column (GE Healthcare, Chicago, USA) and SEC using a 26/60 Superdex 75 column (GE Healthcare, Chicago, USA) and 20 mM acetate buffer pH 5.0, 100 mM NaCl as mobile phase.

SDS-PAGE were performed to assess purity of the protein *T*/SWO. Electrophoretically separated on a 12% SDS-PAGE and visualized by Coomassie Blue staining. Protein concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Rockford, USA) and the Bradford protein assay kit (Bio-Rad).

***T*/SWO activity assays**

The activity of *T*/SWO was measured using the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). Enzyme activity was assayed in a final volume of 1.5 mL, with 1% (w/v) barley β-glucan (Megazyme Co., Bray, Ireland), lichenan (Megazyme Co., Bray, Ireland), laminarin (Megazyme, Wicklow, Ireland) and carboxymethyl cellulose sodium (CMC-Na) (Sigma-Aldrich, St. Louis, MO) as the substrates, and 10 µg/mL of enzyme at optimal conditions for 10 min. The effect of pH was studied using 100 mM citric acid-Na₂HPO₄ (pH 3.0–7.0) buffers at 50 °C and the effect of temperature was evaluated by incubation at pH 4.0, between 30 °C and 90 °C.

Effect of pH and temperature on *T*/SWO activity

Further, the effects of pH and temperature on the activities of *T*/SWO were measured and compared. To determine the optimum pH of *T*/SWO, the activities were assayed with 1% lichenan (w/v) in buffer of different pH, 100 mM glycine-HCl (pH 1.0–3.0), McIlvaine buffer (pH 3.0–8.0) and glycine–NaOH (pH 9.0–12.0). For pH stability, *T*/SWO was preincubated at 37 °C for 1 h in buffers of different pH (1.0–12.0) and subjected to the residual activity assay. For the optimum temperature of *T*/SWO was determined at optimal pH over the temperature range from 30 °C to 80 °C. The thermostability assay of *T*/SWO (100 µg/mL) was carried out by preincubating at 37 °C, 50 °C, 60 °C or 70 °C for 0–60 min, and aliquots of 100 µL were withdrawn at different time points for residual activity assay.

Light and scanning electron microscopic analyses

Avicel PH-101 was used as a solid cellulosic substrate. Ten milligrams of Avicel were incubated with different amount of purified *T*/SWO in 100 mM citric acid- Na_2HPO_4 buffer (pH 4.0). The experiment was carried out on a rotary shaker at 40 °C for different time intervals. Control experiments without *T*/SWO were also performed under the same conditions. The physical structure of Avicel fibers was initially observed using light microscopy (Olympus TH4-200, Japan). Subsequently, photomicrographs of the samples were captured using a scanning electron microscope (Hitachi SU8010, Tokyo, Japan) at a voltage of 15 kV.

Polysaccharide depolymerization analysis

Hydrolysis reactions on 1% barley β -glucan, 1% CMC-Na, 1% laminarin and 0.5% lichenan, were carried out overnight at pH 4.0, 37 °C using the enzyme in a final concentration of 100 µg/mL. High-performance anion-exchange chromatography (HPAEC) (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a Carbo-Pac PA200 column (3×250 mm) was used to determine the reaction products released from the polysaccharide.

Synergism between *T*/SWO and cellulases

The substrate used in this work were NREL dilute acid pre-treated corn stover (PCS) P120927, cellulose nanocrystals (CNCs), phosphoric acid swollen cellulose (PASC) and each substrate equivalent to 8.5 mg of glucan. For CNC preparations from Avicel, about 2 g of Avicel were added to pre-heated HCl at 80 °C. Then run the acid hydrolysis for 4 hours, stirring every 15 minutes with a glass or Teflon rod followed by centrifugation several times, 1600×g for 10 minutes. Decant supernatant and resuspend pellet in DI water until the pH reached 5.0. The CNCs were then suspended in the supernatant after centrifugation. Collect the translucent supernatant and resuspend pellet in DI water and shake vigorously to break up clumps until the supernatant is no longer translucent. The reaction mixtures were carried out in triplicate vials at 40 °C and each substrate was suspended in 20 mM sodium acetate buffer, pH 5.0. The enzyme cocktail comprised endoglucanase I from *Trichoderma longibrachiatum* (Megazyme Co., Bray, Ireland), cellobiohydrolases Cel7A from *Penicillium funiculosum* and β -glucosidase from *Aspergillus niger* (Megazyme Co., Bray, Ireland) at a concentration (mg protein/g of glucan) of 2, 13 and 1, respectively. The reaction was followed for 120 h, with sampling every 24 h. 100 µL samples containing both solids

and liquid are removed from the mixtures and diluted for sugar analysis by high performance liquid chromatography (HPLC) with a BioRad HPX-87H column. Control experiments using BSA were also performed under the same conditions as mentioned above.

Abbreviations

CMC-Na

carboxymethylcellulose sodium

CBM

carbohydrate binding module

GH

glycoside hydrolase

PCS

pre-treated corn stover

CNC

cellulose nanocrystals

PASC

phosphoric acid swollen cellulose

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HZ and YW performed the experiments and performed the experiments. FZ and RB designed and performed the synergism experiments and analyzed the data. BY and XX designed the research and participated in the bioinformatics analysis. FZ and HL revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Abbreviations

CMC-Na: carboxymethylcellulose sodium

CBM: carbohydrate binding module

GH: glycoside hydrolase

PCS: pre-treated corn stover

CNC: cellulose nanocrystals

PASC: phosphoric acid swollen cellulose

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Additional Files

Fig. S1 Production and purification of recombinant *T*/SWO in *Pichia pastoris* and *Trichoderma reesei*. A: SDS-PAGE analysis of the culture supernatant of *T*/SWO produced by *Pichia pastoris*. Lane 1: the crude enzyme of *T*/SWO; lane 2, the purified *T*/SWO; lane 3, the deglycosylated *T*/SWO; B: SDS-PAGE analysis of the culture supernatant of *T*/SWO produced by *Trichoderma reesei*. Lane 1: the crude enzyme of positive control contained *Tr*Cel7; lane 2, the crude enzyme of *T*/SWO; lane 3, the purified *T*/SWO.

Fig. S2 MALDI-TOF-MS analysis of the recombinant protein *T*/SWO.

Fig. S3 Scanning electron microscopic analyses of Avicel. Ten milligrams of Avicel was incubated with 300 μ g of purified *T*/SWO in 100 mM citric acid- Na_2HPO_4 buffer (pH 4.0) for 12 hours.

Figures

Figure 1

Sequence alignments of TlSWO with other 5 swollenins from fungi. AfSWO from *Aspergillus fumigatus* (GeneBank No. XP_747748), PdSWO from *Penicillium decumbens* (GeneBank No. ACH57439), PoSWO from *Penicillium oxalicum* (GeneBank No. ADZ74267), TrSWO from *Trichoderma reesei* (GeneBank No. CAB92328), TpSWO from *Trichoderma pseudokoningii* (GeneBank No. ABV57767.1).

Figure 1

Sequence alignments of TISWO with other 5 swollenins from fungi. AfSWO from *Aspergillus fumigatus* (GeneBank No. XP_747748), PdSWO from *Penicillium decumbens* (GeneBank No. ACH57439), PoSWO from *Penicillium oxalicum* (GeneBank No. ADZ74267), TrSWO from *Trichoderma reesei* (GeneBank No. CAB92328), TpSWO from *Trichoderma pseudokoningii* (GeneBank No. ABV57767.1).

Figure 2

The substrate specificity of the TISWO. Hydrolysis reactions on 1% barley β-glucan, 1% CMC-Na, 1% laminarin and 0.5% lichenan, were carried out overnight at pH 4.0, 50 °C. The linkage type of each substrate was labeled.

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Figure 3

Hydrolysis capacity of TISWO to degrade CMC-Na, lichenan, barley β -glucan and laminarin. All the reactions were carried out overnight at pH 4.0, 37 °C using the enzyme in a final concentration of 100 μ g/mL.

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Hydrolysis capacity of TISWO to degrade CMC-Na, lichenan, barley β -glucan and laminarin. All the reactions were carried out overnight at pH 4.0, 37 °C using the enzyme in a final concentration of 100 μ g/mL.

Figure 4

Enzyme properties of purified recombinant TISWO. (A) pH-activity profile tested at 50 °C. (B) pH stability. After incubation of the enzymes at 37 °C for 1 h in buffers ranging from pH 1.0 to 12.0, the residual activities were determined at pH 4.0 and 50 °C. (C) Temperature-activity profile tested at the pH 4.0. (D) Thermostability. TISWO was pre-incubated at 37 °C, 50 °C, 60 °C and 70 °C for different periods of time, and subjected to residual activity assay under optimal conditions.

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Figure 5

Light microscopic analyses of Avicel. Ten milligram of Avicel was incubated with different amount of purified TISWO in 100 mM citric acid- Na_2HPO_4 buffer (pH 4.0) for 24 hours. (A) 0 μg TISWO (B) 10 μg TISWO, (C) 100 μg TISWO, (D) 300 μg TISWO.

Figure 5

Light microscopic analyses of Avicel. Ten milligram of Avicel was incubated with different amount of purified TISWO in 100 mM citric acid- Na_2HPO_4 buffer (pH 4.0) for 24 hours. (A) 0 μg TISWO (B) 10 μg TISWO, (C) 100 μg TISWO, (D) 300 μg TISWO.

Figure 6

Conversion performance of TISWO on PCS. PCS hydrolysis performed at 40 °C, β -D-glucosidase and cellobiohydrolases were added in the incubation mixture as described in Methods.

Figure 6

Conversion performance of TISWO on PCS. PCS hydrolysis performed at 40 °C, β -D-glucosidase and cellobiohydrolases were added in the incubation mixture as described in Methods.

Figure 7

Conversion performance of TISWO. (A) PASC hydrolysis, (B) CNC hydrolysis. All the reaction performed at 40 °C, β -D-glucosidase, cellobiohydrolases, and endoglucanase were added in the incubation mixture as described in Methods.

Figure 7

Conversion performance of TISWO. (A) PASC hydrolysis, (B) CNC hydrolysis. All the reaction performed at 40 °C, β -D-glucosidase, cellobiohydrolases, and endoglucanase were added in the incubation mixture as described in Methods.

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