Expression of Trichoderma reesei Chitinase and Compositional and Structural Analysis of Its Hydrolysis Products: Postprint

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Abstract

The chitinase gene from Trichoderma reesei was optimized and fully synthesized, and secretory expression was achieved in Pichia pastoris. The secreted chitinase reached a protein concentration of 0.17 mg/mL, with an optimal pH of 5.6, optimal temperature of 65 °C, and enzyme activity of 0.52 U/mL. The enzyme exhibited relatively high stability at temperatures up to 50 °C. The enzyme was employed to hydrolyze low-deacetylation-degree chitosan, and the composition and structure of the resulting products were subsequently analyzed. Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS) analysis revealed that the enzymatic hydrolysis products contained at least 41 chitooligosaccharide components with degrees of polymerization ranging from 2 to 18 and varying deacetylation degrees; nuclear magnetic resonance (NMR) analysis demonstrated that the reducing ends of the product chitooligosaccharides were primarily N-acetylglucosamine, while the non-reducing ends contained both N-acetylglucosamine and glucosamine. These findings may serve as a reference for investigating the structure-function relationships of chitooligosaccharides.

Full Text

Preamble

Expression of Chitinase from *Trichoderma reesei* and Analysis of the Composition and Structure of its Hydrolysates

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Abstract: The chitinase-encoding gene from *Trichoderma reesei* was optimized, synthesized, and secretorily expressed in *Pichia pastoris*. The protein concentration of the expressed chitinase reached 0.17 mg/mL, with an optimal pH of 5.6 and optimal temperature of 65 °C, achieving an enzyme activity of 0.52 U/mL. The enzyme demonstrated good stability at temperatures up to 50 °C. The chitinase was used to hydrolyze low-deacetylation-degree chitosan, and the composition and structure of the products were analyzed. Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS) detection and analysis revealed that the enzymatic hydrolysates contained at least 41 chitooligosaccharide components with degrees of polymerization (DP) ranging from 2 to 18 and varying degrees of deacetylation. Nuclear magnetic resonance (NMR) analysis showed that the reducing ends of the chitooligosaccharide products were primarily composed of N-acetylglucosamine, while the non-reducing ends contained both N-acetylglucosamine and glucosamine. These results provide a valuable reference for investigating the structure-function relationships of chitooligosaccharides.

Keywords: *Trichoderma reesei*; chitinase; *Pichia pastoris*; chitooligosaccharides; UPLC-QTOF MS; NMR

Chitooligosaccharides are composed of glucosamine (GlcN, D) and N-acetylglucosamine (N-acetylglucosamine, A) with degrees of polymerization (DP) typically less than 20. These compounds are usually obtained through physical, chemical, or biological degradation of chitin or its deacetylated derivative chitosan, and have been demonstrated to possess various biological activities including anti-inflammatory, anti-tumor, and plant immune activation effects. Mechanistic studies suggest that chitooligosaccharides exert their activities by interacting with specific receptors in animals and plants. To date, several receptors capable of specifically recognizing chitooligosaccharides have been identified, such as the mannose receptor in animals, chitinase-like binding protein YKL-39, and plant chitin receptors. A common feature of these receptors is their specific recognition of the A sugar unit in chitooligosaccharides, highlighting the critical role of this monosaccharide in biological activity.

Traditional large-scale preparation of chitooligosaccharides typically employs chitosan with high degrees of deacetylation (DDA > 85%) as substrate, using non-specific commercial enzymes with chitosan-hydrolyzing activity such as cellulase, protease, and lipase. The resulting chitooligosaccharides contain minimal residual A sugar, which may impair effective binding to specific receptors and thus limit full activity expression. Additionally, non-specific commercial enzymes may contain multiple chitosan-hydrolyzing activities, leading to uncontrollable product structures that hinder structure-function relationship studies and impede development of novel high-activity chitooligosaccharide products.

Therefore, improving existing preparation processes to obtain structurally controllable chitooligosaccharides with enhanced bioactivity is necessary.

To maximize retention of the key A sugar active unit, low-deacetylation-degree chitosan must be used as substrate. Since such substrates contain both D and A sugars, they can be effectively hydrolyzed by both chitosanases and chitinases. By exploiting differences in substrate recognition between these enzyme classes, chitooligosaccharides with controlled structural features—including DP, DDA, and even acetylation site distribution—can be prepared. Current research on chitooligosaccharide preparation has focused primarily on expression and characterization of chitosanases for hydrolyzing high-DDA chitosan to obtain simple chitooligosaccharide mixtures. In this study, we employed the widely used *Pichia pastoris* expression system for industrial and food enzyme production to secretorily express a chitinase from *Trichoderma reesei*, which was then used to hydrolyze low-DDA chitosan for preparing novel structured chitooligosaccharides whose composition and structural features were characterized.

Materials and Reagents

Plasmid extraction kits, gel recovery kits, T4 ligase, DNA markers, and chemically competent *E. coli* DH5α cells were purchased from Takara Bio (Beijing). The *Pichia pastoris* expression vector pPIC9 was obtained from Youbao Bio, while pGBG1 is a modified vector with pPIC9 signal peptide sequences optimized according to *P. pastoris* codon preferences. *Pichia pastoris* GS115, restriction enzymes Xho , Not , Bgl , and HPLC-grade acetonitrile were purchased from Thermo Fisher Scientific (USA). Preparation methods for *P. pastoris* culture media MD, BMGY, and BMMY, as well as protocols for preparing electrocompetent *P. pastoris* cells, followed the *Pichia pastoris* Expression Manual (Thermo Fisher Scientific). Chitin was purchased from Sigma-Aldrich. Protein markers were obtained from Beijing Solarbio Science & Technology. All other reagents were of analytical grade.

Instruments and Equipment

L535R low-speed refrigerated centrifuge (Xiangyi Centrifuge Instrument Co., Ltd.); RE-2000B rotary evaporator (Gongyi Yingyu High-tech Instrument Factory); LGJ-10FD vacuum freeze dryer (Beijing Songyuan Huaxing Technology Development Co., Ltd.); DYY-6C nucleic acid and protein electrophoresis system (Beijing Liuyi Biotechnology Co., Ltd.); Tanon-1600 gel imaging system (Shanghai Tianneng Technology Co., Ltd.); MicroPulser™ electroporator (Bio-Rad, USA); ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 m); Waters XEVO G2-S QTOF mass spectrometer with Lock-spray interface; electrospray ionization (ESI) source; and MassLynx software.

Experimental Methods

Gene Sequence Optimization, Synthesis, and Expression Vector Construction

The potential chitinase gene from *Trichoderma reesei* QM6a (GenBank: XP_{006968137}.1, encoding amino acids 23–424) was codon-optimized for *Pichia pastoris* preference and Xho and Not restriction sites were added at the termini. The optimized gene was synthesized by Beijing Tsingke Biotech Co., Ltd. The target gene was recovered from pUC18 using Xho and Not and ligated into similarly digested pGBG1 vector, then transformed into *E. coli* DH5α. The constructed expression vector was verified by restriction enzyme digestion and sequencing.

Chitinase Expression and Characterization in *Pichia pastoris*

The recombinant plasmid was linearized with Bgl and the fragment containing the target gene was electroporated into *P. pastoris* GS115. Transformants were selected on MD plates and transferred to BMMY plates containing 0.5% colloidal chitin to screen for the strain producing the largest hydrolysis zone. The selected strain was cultured in BMGY medium and induced with methanol for 120 h. Protein expression in the induced supernatant was detected by SDS-PAGE, and protein concentration was determined by the Bradford method. Enzyme activity against chitosan with 62% DDA (prepared previously) was measured using the DNS method. Optimal pH was determined across the range of 3.6–8.0 (50 mmol/L sodium acetate buffer for pH 3.6–5.6; 50 mmol/L phosphate buffer for pH 6.0–8.0), and optimal temperature was determined from 30 °C to 90 °C. Thermal stability was assessed by measuring residual activity at 40 °C, 50 °C, and 60 °C over 1 h with measurements every 20 min.

Preparation and Component Analysis of Low-Deacetylation-Degree Chitooligosaccharides

The expressed chitinase was used to hydrolyze 62% DDA chitosan as follows: 50 g chitosan was dissolved in 1000 mL water with acetic acid, adjusted to pH 6.0, then 100 mL enzyme solution was added and the mixture was stirred at 40 °C for 48 h. After centrifugation to remove insoluble material, the supernatant was concentrated to approximately 300 mL using a rotary evaporator and lyophilized. For analysis, 10 mg of lyophilized chitooligosaccharide sample was dissolved in ultrapure water to prepare a 1 mg/mL solution. UPLC conditions: Waters ACQUITY UPLC BEH Amide column; mobile phase of 0.1% formic acid in water (A) and acetonitrile (B) with gradient elution (0–2 min: 15% A; 2–32 min: 15–50% A; 32–33 min: 50–80% A; 33–36 min: 80% A; 36–37 min: 80–15% A; 37–44 min: 15% A); column temperature 35 °C; flow rate 0.3 mL/min; injection volume 1 L. MS conditions: ESI source in positive ion mode; capillary voltage 3 kV; cone voltage 60 V; source temperature 150 °C; desolvation temperature 500 °C; cone gas flow 50 L/h; desolvation gas flow 800 L/h; collision energy 30–

60 V; ion energy 3 V; acquisition rate 1 spectrum per 0.25 s; mass range 150–2000 m/z. Data were analyzed using MassLynx 4.1 software to obtain potential chitooligosaccharide mass spectral information and predict composition.

Structural Analysis of Low-Deacetylation-Degree Chitooligosaccharides

The terminal structural features of the hydrolyzed chitooligosaccharides were analyzed using ^{1}H NMR and ^{13}C NMR. Briefly, 25 mg of lyophilized chitooligosaccharide sample was dissolved in 0.50 mL D_2O and analyzed after complete dissolution. Using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as internal standard, samples were analyzed on an AVANCE III 600 MHz NMR spectrometer. Water signal suppression was applied during ^{1}H NMR detection to minimize interference. Raw data were processed using MestReNova software, chemical shifts were calibrated using the internal standard, and specific monosaccharide residues were assigned following the method of Sasaki et al., with reference shifts including reducing-end D sugar (5.19 ppm) and A sugar (5.43 ppm) in ^{1}H NMR, and non-reducing-end D (79.0 ppm) and A (78.5 ppm) C5 carbons in ^{13}C NMR.

Results and Discussion

Expression and Characterization of Chitinase in *Pichia pastoris*

The *T. reesei* QM6a chitinase gene (GenBank: XP_{006968137}.1) spans 1,272 bp, encoding a 424-amino-acid protein with a 22-amino-acid N-terminal signal peptide. The codon-optimized gene was designated *trchi18* (GenBank: MG595778). Restriction digestion verified the constructed expression vector: double digestion with Xho and Not produced a band between 1,000–1,500 bp (E1) matching the target gene size (1,242 bp); Bgl linearization yielded two expected fragments (E2), with the ~10 kb fragment containing the target gene and the ~3 kb fragment containing the resistance gene [Figure 1a: see original paper]. Terminal sequencing confirmed correct insertion of the target gene into the expression vector. SDS-PAGE analysis revealed a prominent protein band near 48 kDa (P1), slightly larger than the predicted molecular weight (44.7 kDa) [Figure 1b: see original paper]. The crude protein concentration in the fermentation supernatant was 0.17 mg/mL by Bradford assay. DNS characterization of the crude chitinase showed optimal activity at pH 5.6 and 65 °C, with specific activity of 0.52 U/mL under these conditions. The enzyme was stable at temperatures up to 50 °C, showing no significant activity loss after 1 h, but was rapidly inactivated at 60 °C.

T. reesei is widely used for industrial production of cellulases, xylanases, β-glucanases, and esterases. Our previous studies revealed that commercial cellulase preparations from *T. reesei* exhibited chitosan-hydrolyzing activity, and structural analysis of the hydrolysates suggested that chitinases were primarily responsible. Seidl et al. identified at least 18 potential chitinase genes in the *T. reesei* genome, indicating abundant chitinase presence. This study expressed

a potential chitinase from *T. reesei* QM6a for chitooligosaccharide preparation. In related work, Ike et al. cloned, expressed in *E. coli*, and characterized the most homologous chitinase from *T. reesei* PC-3-7, finding the native enzyme at ~46 kDa versus a predicted ~42 kDa, suggesting glycosylation. Our expressed chitinase Trchi18 also appeared larger than predicted, likely due to similar glycosylation during expression.

Composition Analysis of Trchi18 Hydrolysates

UPLC-QTOF MS analysis of Trchi18 hydrolysates from low-DDA chitosan showed partial separation of the hydrolysis products [Figure 2a: see original paper]. Sixteen representative peaks (A–P) were selected, and mass spectral analysis identified 41 distinct chitooligosaccharide components [Figure 2b: see original paper]. Based on MS data, these components with various m/z ratios were predicted to be chitooligosaccharides with DP 2–18 and varying DDA . Comparison of total ion chromatograms with MS data revealed that chitooligosaccharides eluted according to increasing D sugar content. When different A sugar contents were present at the same D sugar number, components with D sugar counts $ $3 could be separated, while those with $ $4 D sugars could not be resolved.

Traditional chitooligosaccharide preparation uses high-DDA chitosan (DDA >85%) as substrate, requiring chitosanases or non-specific enzymes with chitosanase activity for effective hydrolysis. At lower DDA values, more A sugars are retained, allowing hydrolysis by chitinases or non-specific enzymes with chitinase activity. Sorbotten et al. used chitinase from *Serratia marcescens* to hydrolyze chitosans with varying DDA, demonstrating that DP distribution changed with DDA. This suggests that controlling DDA enables regulation of product DP. Our approach using chitinase to hydrolyze low-DDA chitosan yielded products with broader DP distribution (2–20) compared to traditional methods (primarily DP <10), potentially conferring higher bioactivity.

Structural Analysis of Trchi18 Hydrolysates

To determine the substrate site recognition characteristics of Trchi18 and structural features of the products, ^{1}H NMR and ^{13}C NMR were used to analyze terminal structures of the chitooligosaccharide hydrolysates. The results showed that reducing ends were predominantly composed of A sugar [Figure 3a: see original paper], while non-reducing ends contained both D and A sugars [Figure 3b: see original paper], indicating that the enzyme can hydrolyze both A–A and A–D glycosidic bonds. Seidl et al. previously analyzed 18 potential chitinases from *T. reesei* QM6a and found all belonged to glycoside hydrolase family 18, whose characteristic feature is the requirement for an A unit at the -1 subsite, theoretically yielding A sugar at the reducing end. Our NMR results confirm that the expressed chitinase belongs to glycoside hydrolase family 18.

Conclusion

In summary, this study successfully secretorily expressed a chitinase gene from *Trichoderma reesei* in *Pichia pastoris* and used the enzyme to hydrolyze low-deacetylation-degree chitosan, with subsequent analysis of chitooligosaccharide composition and structural features. Compared to traditionally prepared chitooligosaccharides, those produced by *T. reesei* chitinase hydrolysis of low-DDA chitosan exhibit greater component diversity and controllable terminal structures, potentially offering higher bioactivity and broader application prospects.

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Note: Figure translations are in progress. See original paper for figures.