Postprint: Preparation of Low-Degree-of-Deacetylation Chitooligosaccharides by Bacillus subtilis Chitosanase Hydrolysis and Component Analysis

Authors: Cheng Gong, Jiao Siming, Ren Lishi, Feng Cui, Du Yuguang

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Abstract

The chitosanase-encoding gene derived from Bacillus subtilis strain 168 was subjected to sequence optimization and complete synthesis, and secretory expression was achieved in Pichia pastoris, with the protein concentration of the expressed product reaching 0.30 mg/ml. The expressed chitosanase exhibited an optimal pH of 5.6, an optimal temperature of 55 °C, and a specific activity of 84.54 U/ml. The enzyme demonstrated relatively high stability at temperatures of 50 °C and below. Low-deacetylation-degree chitosan was hydrolyzed using this enzyme, and the product components were separated and identified by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS). Based on the primary mass spectrometry data, the enzymatic hydrolysis products were inferred to contain at least 37 types of chitooligosaccharide components with degrees of polymerization ranging from 2 to 18 and varying degrees of deacetylation. In summary, the chitosanase gene derived from Bacillus subtilis strain 168 was secretively expressed in Pichia pastoris, and the expressed product was utilized to hydrolyze and prepare low-deacetylation-degree chitooligosaccharides with their components analyzed, which may provide a reference for subsequent studies on the structure-function relationships of chitooligosaccharides.

Full Text

Preparation and Composition Analysis of Chitooligosaccharides with Low Degree of Deacetylation by Hydrolysis of Bacillus subtilis Chitosanase

CHENG Gong, JIAO Si-ming, REN Li-shi, FENG Cui, DU Yu-guang

State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

Abstract

The chitosanase encoding gene from *Bacillus subtilis* 168 was codon-optimized, synthesized, and secretorily expressed in *Pichia pastoris*. The protein concentration of the expressed product reached 0.30 mg/ml. The expressed chitosanase exhibited optimal activity at pH 5.6 and 55 °C, with a specific activity of 84.54 U/ml. The enzyme demonstrated good stability at temperatures up to 50 °C. Low-deacetylation chitosan was hydrolyzed using this enzyme, and the product composition was analyzed via ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS). Based on primary mass spectrometry data, the hydrolysates were predicted to contain at least 37 distinct chitooligosaccharide components with degrees of polymerization (DP) ranging from 2 to 18 and varying degrees of deacetylation. In summary, chitosanase from *Bacillus subtilis* 168 was secretorily expressed in *Pichia pastoris* and used to prepare low-deacetylation chitooligosaccharides, whose composition was analyzed to provide a reference for future studies on the structure-function relationships of chitooligosaccharides.

Keywords: *Bacillus subtilis*; chitosanase; *Pichia pastoris*; chitooligosaccharides; ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS)

Chitooligosaccharides are composed of glucosamine (GlcN, D) and *N*-acetylglucosamine (*N*-acetylglucosamine, A) with a degree of polymerization (DP) less than 20 [1]. Studies have demonstrated that chitooligosaccharides possess various biological activities, including anti-inflammatory, antioxidant, antitumor, and plant immune activation properties [2-5]. Structural characteristics such as DP, degree of deacetylation (DDA), and the distribution of A sugar units along the oligosaccharide chain may significantly influence their biological activity [6].

In industrial applications, chitooligosaccharides are typically prepared by degrading high-deacetylation chitosan using biological, physical, or chemical methods. Since most A sugar units, which serve as potential active components, are converted to D sugars during chitosan preparation, the resulting chitooligosaccharides exhibit limited structural diversity, thereby restricting their full biological activity [7-8]. Moreover, due to the lack of highly specific chitosanases, non-specific commercial enzymes with chitosan-hydrolyzing activity—such as cellulase [9], protease [10], and lipase [11]—are commonly employed in enzymatic preparation. These commercial enzymes may contain multiple chitosan-degrading enzymes, including chitosanases and chitinases, yielding hydrolysates with complex structures that make it difficult to obtain chitooligosaccharides with controllable structures and hinder structure-function relationship studies.

To address these challenges in industrial preparation, application, and basic research of chitooligosaccharides, breakthroughs are needed in three key areas: (1) obtaining efficient and specific chitosan-hydrolyzing enzymes for large-scale preparation; (2) preserving A sugar units to the greatest extent during chitosan preparation to produce low-deacetylation chitosan, thereby maximizing biological activity; and (3) utilizing specific and efficient chitosan hydrolases with different substrate specificities to hydrolyze low-deacetylation chitosan, producing multi-component, structurally complex chitooligosaccharides with controllable structures for subsequent separation, identification, evaluation of activity differences, and mechanistic studies to ultimately elucidate structure-function relationships and molecular mechanisms. Following this approach, we have conducted preliminary work: we achieved efficient secretory expression of a chitosanase gene from *Bacillus subtilis* in *Pichia pastoris*, and used the enzyme to prepare low-deacetylation chitooligosaccharides with preliminary separation and compositional analysis.

Materials and Reagents

Pichia pastoris GS115 (Cat. No. C18100), restriction enzymes Xho (Cat. No. FD0695), Not (Cat. No. FD0596), Bgl (Cat. No. FD0084), and HPLC-grade acetonitrile (Cat. No. A998-4) were purchased from Thermo Fisher Scientific (USA). Plasmid extraction kit (Cat. No. 9760), gel recovery kit (Cat. No. 9762), T4 DNA ligase (Cat. No. 2011A), and *E. coli* DH5 chemically competent cells (Cat. No. 9057) were obtained from Takara Bio (Beijing). The *Pichia pastoris* expression vector pPIC9 (Cat. No. VT1343) was purchased from YouBio, while pGBG1 is a vector optimized for *Pichia pastoris* codon preference based on the pPIC9 signal peptide sequence [12]. Preparation methods for *Pichia pastoris* culture media MD, BMGY, and BMMY, as well as electrocompetent cell preparation, followed the *Pichia pastoris* Expression Manual (Cat. No. K1740-01, Thermo Fisher Scientific, USA). Protein marker (Cat. No. PR1920) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Chitin (Cat. No. V900332) was obtained from Sigma-Aldrich. All other reagents were of analytical grade.

Instruments and Equipment

L535R low-temperature refrigerated centrifuge (Xiangyi Centrifuge Instrument Co., Ltd.); DYY-6C nucleic acid and protein electrophoresis system (Beijing Liuyi Biological Technology Co., Ltd.); Tanon-1600 gel imaging system (Shanghai Tanon Science & Technology Co., Ltd.); MicroPulser™ electroporator (Bio-Rad, USA); RE-2000B rotary evaporator (Gongyi Yingyu High-Tech Instrument Factory); LGJ-10FD vacuum freeze dryer (Beijing Songyuan Huaxing Technology Development Co., Ltd.); ACQUITY UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 m); Waters XEVO G2-S QTOF mass spectrometer equipped with Lock-spray interface, electrospray ionization (ESI) source, and MassLynx 4.1 software (Waters, USA).

Methods

Codon Optimization, Synthesis, and Expression Vector Construction of *Bacillus subtilis* Chitosanase Gene

The chitosanase encoding gene from *Bacillus subtilis* 168 [13] (GenBank: CAB14630, amino acids 36-277, signal peptide sequence removed) was selected for codon optimization based on *Pichia pastoris* preference without altering the amino acid sequence. Xho and Not restriction sites were added to the 5' and 3' ends, respectively. The optimized sequence was synthesized by Beijing Tsingke Biotech Co., Ltd. Both the pUC18 plasmid containing the target gene and the expression vector pGBG1 were double-digested with Xho and Not . The target gene fragment and linearized pGBG1 vector were recovered and ligated using T4 DNA ligase, then transformed into *E. coli* DH5 chemically competent cells. The constructed expression plasmid was verified by restriction enzyme digestion and sequencing (performed by Beijing Tsingke Biotech Co., Ltd.).

Expression and Preliminary Characterization of *Bacillus subtilis* Chitosanase in *Pichia pastoris*

The recombinant plasmid was linearized with Bgl , separated by gel electrophoresis, and the large fragment containing the target gene was recovered and electroporated into freshly prepared *Pichia pastoris* GS115 electrocompetent cells. Recombinants were selected on MD plates lacking histidine, and a number of colonies were streaked onto BMMY agar plates containing 0.5% colloidal chitosan. After incubation at 30 °C for 2-3 days, the clone with the largest hydrolysis zone was selected. The selected single colony was inoculated into 200 ml BMGY medium and cultured at 28 °C, 250 rpm for 48 h. Cells were harvested by centrifugation, the supernatant was discarded, and 200 ml BMMY medium was added for induction. Methanol was supplemented to a final concentration of 1% after 24 h and every 24 h thereafter. After 120 h of induction, cells were removed by centrifugation and the supernatant was collected as crude enzyme solution. Protein expression in the fermentation supernatant was analyzed by SDS-PAGE, and protein concentration was determined by the Bradford method. Enzyme activity was measured using the DNS method with prepared 62% deacetylated chitosan [14] as substrate. Optimal pH was determined in the range of 3.6-8.0 (50 mM sodium acetate buffer for pH 3.6-5.6; 50 mM phosphate buffer for pH 6.0-8.0), and optimal temperature was determined between 30 °C and 90 °C. Thermostability was assessed by incubating the chitosanase at 40 °C, 50 °C, and 60 °C for 1 h, measuring residual activity every 20 min.

Preparation and Composition Analysis of Low-Deacetylation Chitooligosaccharides

Low-deacetylation chitosan prepared previously [14] (62% DDA) was hydrolyzed to prepare chitooligosaccharides. Fifty grams of low-deacetylation chitosan was

dissolved in 1000 ml water with acetic acid, adjusted to pH 6.0, and 10 ml crude enzyme solution was added. The reaction was carried out at 40 °C with stirring for 48 h. After centrifugation to remove insoluble residues, the supernatant was concentrated to approximately 300 ml using a rotary evaporator and lyophilized. Ten milligrams of the 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.

Mass spectrometry conditions: ESI source in positive ion mode; capillary voltage 3 kV; cone voltage 60 V; source temperature 150 °C; desolvation gas temperature 500 °C; cone gas flow 50 L/h; desolvation gas flow 800 L/h; collision energy 30-60 V; ion energy 3 V; spectra acquisition every 0.25 s; mass scan range 150-2000 m/z. MassLynx 4.1 software was used to analyze the LC-MS data to obtain potential chitooligosaccharide information and predict their composition.

Results and Discussion

Codon Optimization, Synthesis, and Expression of *Bacillus subtilis* Chitosanase Gene in *Pichia pastoris*

The chitosanase encoding gene from *Bacillus subtilis* 168 is 831 bp in length, encoding a protein of 277 amino acids with a 35-amino-acid signal peptide at the N-terminus. The codon-optimized gene sequence was designated *bscsn* (GenBank: MG595775). Comparison of the original and optimized sequences revealed 195 nucleotide changes. The synthesized gene was cloned into the *Pichia pastoris* expression vector pGBG1 and verified by restriction digestion. Electrophoresis showed that double digestion of the recombinant plasmid with Xho and Not produced a band near 750-1000 bp (E1), consistent with the target gene size (762 bp). Linearization with Bgl yielded two expected fragments (E2): a ~10 kb fragment containing the target gene *bscsn* and a ~3 kb fragment containing the antibiotic resistance gene (Figure 1a [Figure 1: see original paper]). Sequencing of both ends confirmed correct insertion of the target gene into the expression vector.

The expression vector *bscsn*-pGBG1 was transformed into *Pichia pastoris* GS115 and induced with methanol. SDS-PAGE analysis of the expression product (designated BSCSN) revealed two protein bands near 35 kDa and between 25-35 kDa (P1). The 25-35 kDa band matched the predicted molecular weight (28.3 kDa), while the 35 kDa band was hypothesized to be a glycosylated product (Figure 1b [Figure 1: see original paper]). The protein concentration in the crude enzyme solution was determined to be 0.30 mg/ml by the Bradford method.

Previous studies have reported on the chitosanase from *Bacillus subtilis* 168:

Parro et al. [15] first identified the potential chitosanase coding gene during genome sequencing; Rivas et al. [16] expressed this chitosanase in *B. subtilis* and characterized its enzymatic properties; Pechsrichuang et al. [17] fused the chitosanase gene with the *E. coli* OmpA signal peptide and achieved secretory expression in *E. coli*. This study represents the first codon optimization and secretory expression of this chitosanase gene in *Pichia pastoris*. In addition to strain 168, chitosanase genes from other *B. subtilis* isolates have been expressed and characterized, such as the study by Kang et al. [18] which secretorily expressed the chitosanase gene from strain HD145 in *Pichia pastoris* and analyzed the composition of the resulting chitooligosaccharides.

Preliminary Characterization of Chitosanase BSCSN Enzymatic Properties

Enzyme activity in the fermentation supernatant was measured using the DNS method. The enzyme exhibited optimal activity at pH 5.6 (Figure 2a [Figure 2: see original paper]) and 55 °C (Figure 2b [Figure 2: see original paper]), with a specific activity of 84.54 U/ml under these optimal conditions. Thermostability assays showed that the enzyme remained stable at temperatures up to 50 °C, with no significant activity loss after 1 h of incubation, but was rapidly inactivated at 60 °C (Figure 2c [Figure 2: see original paper]). These results align well with those of Rivas et al. [16], who reported optimal pH and temperature of 5.7 and 60 °C, respectively, and good stability up to 50 °C when the gene was expressed in *B. subtilis*. Pechsrichuang et al. [17] reported optimal pH and temperature of 5.0-6.0 and 40-50 °C, respectively, with stability up to 45 °C and rapid inactivation at 50 °C when expressed in *E. coli*, indicating relatively poor thermostability in that expression system.

Composition Analysis of Chitosanase BSCSN Hydrolysis Products

The secretorily expressed chitosanase BSCSN from *Pichia pastoris* was used to hydrolyze low-deacetylation chitosan substrate (62% DDA). UPLC-QTOF MS detection and analysis showed that the hydrolysis products could be partially separated (Figure 3a [Figure 3: see original paper]). Nine representative peaks (A-I) were selected, and based on their corresponding mass spectra, a total of 37 distinct potential chitooligosaccharide components with different molecular weights were identified (Figure 3b [Figure 3: see original paper]). Analysis of primary mass spectra for these components with different mass-to-charge ratios (m/z) allowed prediction of their monosaccharide composition, degree of polymerization, and deacetylation. These 37 components were predicted to be chitooligosaccharides with DP 2-18 and varying degrees of deacetylation (Table 1).

Correlation of the chitooligosaccharides in Table 1 with Figure 3 revealed that the elution order generally followed the number of glucosamine (D) units, from few to many. This indicates that the current separation conditions can achieve preliminary fractionation of these complex chitooligosaccharide components.

However, further optimization of separation materials and conditions will be necessary to obtain single molecular weight components or even defined structures (such as determining the distribution of A sugars in the oligosaccharide chain).

In the study by Kang et al. [18], hydrolysis products of chitosans with different deacetylation degrees (DDA: 50%, 70%, and 90%) using expressed chitosanase were primarily fully deacetylated chitooligosaccharides with DP < 8, which differs substantially from our results showing numerous partially deacetylated chitooligosaccharides with higher DP. This discrepancy is likely attributable to differences in the chitosan substrates used. Research suggests that binding to specific receptors in animals and plants may be an important mechanism for chitooligosaccharide bioactivity, and multiple receptors that specifically recognize chitooligosaccharide structures have been identified [19-25]. Interestingly, different receptors may interact with different chitooligosaccharide structures to exert distinct biological activities: certain plant chitin receptors require binding to chitin oligosaccharides (fully acetylated chitooligosaccharides) with DP 6 to activate innate immunity [26], while other receptors bind to low-DP oligosaccharides such as chitotetraose and chitopentaose to promote plant growth [27]. Whether these plant receptors can bind to specific partially deacetylated chitooligosaccharides and exert particular biological activities remains to be systematically investigated.

Currently, due to limitations in preparation technology, commercial chitooligosaccharides are primarily prepared from high-deacetylation chitosan, resulting in products that are mainly low-DP (DP < 10), high-DDA (DDA $> 85\%$) chitooligosaccharides with limited component diversity and scarce A sugar-containing oligosaccharides. This restricts receptor binding and limits full expression of their biological activity. In this study, we achieved secretory expression of chitosanase from a safe microbial source (*Bacillus subtilis*) using the *Pichia pastoris* expression system, which can be combined with high-density fermentation for efficient and cost-effective production. Moreover, using this enzyme to hydrolyze scalable low-deacetylation chitosan enables large-scale preparation of low-deacetylation, structurally complex chitooligosaccharides. Future research will focus on these developments.

Conclusion

This study optimized and synthesized a chitosanase gene from *Bacillus subtilis* and achieved its secretory expression in *Pichia pastoris*. The resulting chitosanase was used to hydrolyze low-deacetylation chitosan, and compositional analysis of the hydrolysates revealed the presence of multiple chitooligosaccharides with various degrees of polymerization and deacetylation. Future work will focus on further separation and preparation of these components to enable in-depth investigation of chitooligosaccharide structure-function relationships, providing a foundation for developing and applying novel chitooligosaccharide products with enhanced bioactivity.

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