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Cloning, Expression, and Activity of Chitinase Genes from Endosymbiotic Bacteria of Periplaneta americana (Postprint)

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Abstract

Abstract [Objective] To clone, achieve soluble expression, and functionally validate the chitinase ChiA gene from endophytic Serratia marcescens in Periplaneta americana. [Methods] The ChiA gene was amplified by PCR, subcloned using TA cloning, and used to construct the recombinant expression vector ChiA/pET21b. Bioinformatics analysis, low-temperature induction expression, SDS-PAGE, and Western blot identification were performed, and chitinase activity was detected using the well diffusion method. Results The ChiA gene sequence was successfully amplified by PCR, exhibiting 99% homology with the S. marcescens ChiA gene sequence in GenBank. This sequence encodes 571 amino acids and can be stably expressed in a prokaryotic system. Soluble expression analysis demonstrated that soluble target protein was obtained through low-temperature induction expression. Activity assays revealed that the expression product containing the target protein could degrade chitin, with activity stronger than that of the endophytic Serratia marcescens from Periplaneta americana. [Conclusion] The chitinase ChiA gene was successfully cloned from the genome of endophytic Serratia marcescens in Periplaneta americana, and soluble chitinase ChiA with strong activity was obtained through the prokaryotic expression system, laying a foundation for its application.

Full Text

Cloning, Expression, and Activity Verification of a Chitinase Gene from Endophytic Bacteria in *Periplaneta americana*

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Abstract

[**Objective**] To clone the chitinase ChiA gene from endophytic *Serratia marcescens* in *Periplaneta americana*, achieve soluble expression of the protein, and verify its function. [**Methods**] The ChiA gene was amplified by PCR and subcloned using a TA cloning strategy to construct the recombinant expression vector ChiA/pET21b. Bioinformatics analysis, low-temperature induction, SDS-PAGE, Western blot identification, and a punch-plate assay were performed to detect chitinase activity. **Results** The ChiA gene sequence was successfully amplified by PCR, showing 99% homology with the *S. marcescens* ChiA gene sequence in GenBank. The sequence encoded 571 amino acids and could be stably expressed in a prokaryotic system. Solubility analysis demonstrated that soluble target protein was obtained through low-temperature induction. Activity assays revealed that the expressed product could degrade chitin, with activity stronger than that of the endophytic *S. marcescens* from *P. americana*. [**Conclusion**] The chitinase ChiA gene was successfully cloned from the genome of endophytic *S. marcescens* in *P. americana*, and soluble chitinase ChiA with strong activity was obtained through a prokaryotic expression system, laying a foundation for its future application.

Keywords: *Periplaneta americana*; *Serratia marcescens*; chitinase ChiA; soluble expression; activity verification

Introduction

Chitin, a linear polymer of N-acetyl- -D-glucosamine linked by -1,4-glycosidic bonds, is widely distributed in nature as the second most abundant renewable resource after cellulose [1]. Chitin oligosaccharides, monosaccharides, and their derivatives produced through chitin degradation have broad application prospects in biomedicine, industry, agriculture, and food sectors [3]. Industrial extraction of chitin derivatives typically involves pretreatment with strong acids or bases to remove protein impurities and pigments, followed by chemical, physical, or enzymatic treatment of chitosan to obtain chitin derivatives. Chemical methods often cause environmental pollution and side reactions, while physical methods require sophisticated equipment and produce non-uniform products. Enzymatic hydrolysis offers a more rational and efficient approach with lower equipment requirements, controllable reaction products, and minimal environmental impact [4]. Chitinase (EC 3.2.1.14) was first discovered by Benecke in 1905 during studies on *Bacillus chitinovorous* decomposition of chitin [5]. This enzyme catalyzes the hydrolysis of -1,4-glycosidic bonds in chitin to generate N-acetylglucosamine [6]. Chitinase-producing microorganisms have been isolated from various sources including soil, shellfish waste, gardens, and hot springs [7]. Among these, *Serratia marcescens* is a relatively high-yield chitinase producer that secretes numerous antibiotic metabolites such as lipopeptides, carbapen-

ems, prodigiosin, chitinase, and isothiomycin, effectively inhibiting the growth of various plant pathogenic fungi [8].

In our previous research, we isolated multiple endophytic strains from the gut of *Periplaneta americana*, including *Serratia marcescens* [9] designated as WA12 1-18 (preserved in our laboratory, GenBank accession number: MH341634). Studies have shown that due to the unique endophytic environment within insects, endophytic bacteria may produce different metabolites influenced by this special habitat, with potential medical value, novel molecular structures, and complex biosynthetic pathways [10]. This study cloned the chitinase ChiA gene from endophytic *S. marcescens* in *P. americana*, obtained soluble chitinase through prokaryotic expression, and preliminarily verified its activity, establishing a foundation for further research and application of this strain.

Materials and Methods

1.1 Experimental Materials and Reagents

1.1.1 Bacterial Strains

The experimental strains included *Serratia marcescens* WA12 1-18, *E. coli* DH5 , *E. coli* BL21(DE3), and the pET21b vector (all preserved in our laboratory).

1.1.2 Reagents

LA Taq DNA Polymerase, restriction enzymes XhoI and NdeI, T4 DNA Ligase, pMD18-T vector, and DNA molecular weight markers were purchased from TaKaRa. DNA purification and plasmid extraction kits were from TIANGEN. First-strand synthesis kit was from Invitrogen. Protein prestained marker was from Fermentas. Chitin was from Shanghai Macklin Biochemical Technology. Agarose was from Amresco. His-Tag (IOE2) monoclonal antibody and HRP-labeled goat anti-mouse IgG secondary antibody were from Santa Cruz. Other common reagents were analytical grade from domestic or international sources.

1.2 Experimental Methods

1.2.1 PCR Amplification and TA Cloning of ChiA Gene

Genomic DNA of endophytic *S. marcescens* WA12 1-18 from *P. americana* was extracted using a bacterial DNA extraction kit. Based on the ChiA gene sequence from GenBank (AY855211.1), two specific primers were designed using Primer Premier 5.0 software: Forward primer 5' -CCGGAATTCCATATGCGCAAATTTAATAAACCGCTGTTGG-3' and Reverse primer 5'-GCTCTAGACTCTCGAGTTGAACGCCGGCGCTGTTGCCCAG-3' . NdeI and XhoI restriction sites were introduced at the 5' ends of the forward and reverse primers, respectively, with the stop codon removed. Using genomic DNA as template, the ChiA DNA sequence was amplified with TaKaRa LA Taq under the following conditions: denaturation at 95°C for 30 s, annealing at 60°C for 45 min, and extension at 72°C for 2 min, for 30 cycles. PCR products were detected by agarose gel electrophoresis and ligated into the pMD18-T

vector, then transformed into *E. coli* DH5 competent cells for blue-white screening. White colonies were selected for plasmid extraction and restriction enzyme digestion. Positive recombinant strains ChiA/pMD18-T/DH5 were sent to Shanghai Invitrogen for sequencing.

1.2.2 Construction and Identification of Expression Vector ChiA/pET21b

Correctly sequenced ChiA/pMD18-T and the pET21b expression vector were double-digested with XhoI and NdeI. After recovering the digested products, the ChiA gene fragment was ligated with digested pET21b using T4 ligase and transformed into *E. coli* DH5 competent cells. Transformants were plated on ampicillin-containing plates and cultured for 18 h. Single colonies were selected for colony PCR and plasmid digestion identification. Positive recombinant strains ChiA/pET21b/DH5 were sent to Shanghai Invitrogen for sequencing.

1.2.3 Bioinformatics Analysis of ChiA Gene

The ProtParam tool (http://www.expasy.ch/tools/protparam.html) was used to analyze basic physicochemical parameters of the ChiA-encoded protein, including amino acid composition, molecular weight, theoretical isoelectric point, half-life in mammals, *E. coli*, and yeast, instability index, and aliphatic index. The ProtScale tool (http://expasy.org) was employed to analyze hydrophobicity/hydrophilicity changes of each amino acid in the expressed product.

1.2.4 Induced Expression and Solubility Analysis of ChiA/pET21b/BL21 Recombinant Strain

The correctly sequenced expression vector ChiA/pET21b was transformed into *E. coli* BL21(DE3) competent cells to obtain the ChiA/pET21b/BL21 recombinant strain. A single colony was inoculated into 5 mL LB medium containing 100 g/mL ampicillin and cultured overnight at 37°C with shaking. This culture was then inoculated into fresh LB medium at a 1:100 ratio and grown at 37°C for 2.5 h (OD 0.6). Expression was induced with 0.1 mmol/L IPTG at 20°C for 20 h. Cells were harvested, washed twice with 1×PBS, and resuspended in lysis buffer (10 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 8.0). Cells were lysed by sonication on ice (30% power, 10 s pulse, 15 s interval, 15 min). After centrifugation at 8000 r/min for 15 min, the cell pellet, supernatant, and precipitate were analyzed by SDS-PAGE and Western blot.

1.2.5 Detection of Target Protein Chitinase Activity by Punch-Plate Method

Colloidal chitin was prepared according to established methods [11]: 10 g of powdered chitin was added to 100 mL concentrated HCl, stirred in an ice bath for 30 min, then stored at 4°C for 24 h. While stirring, the mixture was added to a beaker containing 3 L water. After layering, the supernatant was removed, distilled water was added, and the process was repeated 7-8 times. Large particles were filtered through single-layer gauze until the pH reached 6.4. The prepared 10 mL colloidal chitin was mixed with 100 mL melted solid LB medium and poured into plates. Four wells were punched in each plate and labeled 1-4. One hundred microliters of ChiA/pET21b/BL21 recombinant cells, WA12 1-18

cells, ChiA/pET21b/BL21 lysate supernatant, and lysis buffer were added to the wells, respectively.

Results

2.1 Cloning and Identification of ChiA Gene

The ChiA gene sequence from GenBank (AY855211.1) is 1692 bp. Both PCR amplification and ChiA/pMD18-T digestion showed a target band at approximately 1700 bp [Figure 1: see original paper]. Sequencing of positive recombinant strains ChiA/pMD18-T/DH5 revealed that the target sequence differed from *Serratia marcescens* WW4 ChiA (GenBank CP003959.1) by only four base mutations at positions 372, 1479, 1506, and 1552 bp. Due to codon degeneracy, only one sense mutation occurred (Thr→Ala), and both amino acids are uncharged neutral residues, suggesting minimal impact on protein structure.

2.2 Construction and Identification of ChiA/pET21b Expression Vector

Colony PCR and restriction digestion identification of the recombinant expression vector ChiA/pET21b showed a target band at approximately 1700 bp by colony PCR, and two bands at approximately 1700 bp and 5443 bp by double digestion [Figure 2: see original paper]. Sequencing confirmed correct orientation of the target gene in the expression vector, with ORF Finder analysis revealing a 1716 bp open reading frame.

2.3 Bioinformatics Analysis Results

The open reading frame encoded a protein of 571 amino acids [Figure 3: see original paper] with a molecular weight of 62 kD, containing 57 negatively charged residues (Asp+Glu) and 54 positively charged residues (Arg+Lys). The theoretical isoelectric point was 6.47. The protein half-life was 30 h in mammalian reticulocytes, >20 h in yeast, and >10 h in *E. coli*, with an instability index of 14.56. Hydrophilicity/hydrophobicity prediction using ProtScale Server showed that Pro at position 151 had the strongest hydrophilicity (-3.178), while Leu at position 12 and Ile at position 13 had the strongest hydrophobicity (2.178). The number of hydrophilic amino acids exceeded that of hydrophobic amino acids [Figure 4: see original paper].

2.4 Identification and Solubility Analysis of Expression Product

The successfully constructed expression vector ChiA/pET21b was transformed into BL21 for induced expression. SDS-PAGE analysis showed a target band at 62 kD. Western blot using mouse anti-His monoclonal antibody as primary antibody revealed specific bands at 62 kD in both induced total cells and lysate supernatant [Figure 5: see original paper].

2.5 Chitinase Activity Assay Results

Serratia marcescens WA12 1-18, induced ChiA/pET21b/BL21 cells, and lysate supernatant were added to LB solid medium containing chitin, with buffer as blank control. Transparent halos appeared around wells containing bacteria and supernatant, while no halo was observed in the control. The halo diameter produced by lysate supernatant was larger than those produced by ChiA/pET21b/BL21 cells and *S. marcescens* WA12 1-18 [FIGURE:6, TABLE:1].

Discussion

This study successfully cloned the chitinase ChiA gene sequence from endophytic *Serratia marcescens* in the gut of *Periplaneta americana* for the first time. NCBI BLAST comparison revealed that the cloned gene sequence showed over 99% similarity with *Serratia marcescens* ChiA genes from GenBank accessions CP003959.1, Z36294.1, CP021984.1, and KP728831.1. Compared with *S. marcescens* WW4 ChiA (CP003959.1), only four base differences were found: 372 bp (C→T), 1479 bp (T→C), 1506 bp (T→A), and 1552 bp (A→G) (ACC→GCC: Thr→Ala). Due to codon degeneracy, only one sense mutation occurred (Thr→Ala), and both amino acids are uncharged neutral residues with minimal impact on protein structure [12]. Compared with *S. marcescens* (BJL200) chiA gene (Z36294.1), seven base differences were identified at positions 477 bp (G→C), 588 bp (T→C), 703 bp (C→T, CTG→TTG: Leu→Leu), 822 bp (C→T), 1479 bp (T→C), 1506 bp (T→A), and 1518 bp (A→G), with no amino acid changes. These results indicate that the cloned chitinase ChiA gene maintains the conserved structure and biological activity of chitinase A [13], which was verified by subsequent experiments demonstrating chitinase activity of the expressed target protein.

In primer design, the stop codon at the end of the ChiA gene was removed, resulting in a recombinant expression vector ChiA/pET21b open reading frame of 1716 bp comprising the ChiA gene sequence, XhoI site, and the 6×His coding sequence from pET21b, encoding 571 amino acids. Bioinformatics analysis revealed that the encoded protein had a half-life >10 h in *E. coli* and an instability index of 14.56, favorable for prokaryotic expression. The GRAVY index was -0.465, with more hydrophilic than hydrophobic amino acids, indicating hydrophilic properties conducive to soluble expression and subsequent purification [14]. SDS-PAGE and Western blot results confirmed successful expression of ChiA in the prokaryotic system. Activity tests showed that *S. marcescens* WA12 1-18, induced ChiA/pET21b/BL21 cells, and lysate supernatant all produced transparent halos of varying sizes, while the blank control showed no halo. The lysate supernatant produced the largest halo diameter (approximately 24 mm), indicating soluble expression of chitinase. The halo produced by lysate supernatant was larger than those from ChiA/pET21b/BL21 cells (approximately 20 mm) and *S. marcescens* WA12 1-18 (approximately 22 mm). Compared with literature reports where the maximum halo diameter was approximately 28 mm

[15], the soluble chitinase induced from ChiA/pET21b/BL21 demonstrated significant and strong activity.

Although chitinase is widely distributed in nature, its low activity and yield cannot meet the requirements for large-scale chitin and chitooligosaccharide production [16]. *E. coli* plays an important role in biotechnology research and biopharmaceutical industrialization, often selected as an expression tool due to its rapid reproduction, low cost, and high-level target gene expression [17]. We aimed to leverage these characteristics to achieve successful chitinase expression with improved yield and production stability. *Serratia marcescens* is a commonly used high-yield chitinase-producing strain with great application potential in agricultural biocontrol and other fields. Our laboratory first isolated *S. marcescens* from cockroaches, and few reports exist on chitinase activity from cockroach endophytic *S. marcescens*. Considering its special growth environment, it may possess higher chitinase activity than natural isolates. This study combined these advantages to improve chitinase yield, activity, and expression stability. Although Shapira et al. cloned the *S. marcescens* chitinase ChiA gene using pBR322 as a vector in 1989, the expression level was not high [18]. Therefore, this study successfully cloned, expressed solubly, and obtained active chitinase ChiA from cockroach endophytic *S. marcescens*, establishing a foundation for in-depth research and application of chitinase ChiA and providing new insights for chitin utilization and industrial production.

In conclusion, this study successfully cloned the chitinase A gene from cockroach endophytic *Serratia marcescens* by PCR and constructed the prokaryotic expression system ChiA/pET21b/BL21. IPTG induction, SDS-PAGE, and Western blot analysis confirmed successful expression of the target protein. The punch-plate method preliminarily demonstrated that the induced protein possessed chitinase activity stronger than that of *S. marcescens* (WA12 1-18), with greater stability in the supernatant than in *S. marcescens* (WA12 1-18). These results provide a foundation for subsequent studies on the biological activity and industrial development of chitinase.

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