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Expression and characterization of a novel chitinase with antifungal activity from a rare actinomycete, *Saccharothrix yanglingensis* Hhs.015

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ABSTRACT

Saccharothrix yanglingensis Hhs.015, a new type of rare actinomycete, was isolated from the roots of cucumber. A novel chitinase gene was cloned from *S. yanglingensis* Hhs.015 and overexpressed as a soluble protein Chi6769 (77.9 kDa) in *Escherichia coli*. Chi6769 was purified by HisTrap HP affinity chromatography with optimal pH of 7.0. The enzymatic hydrolysis assay revealed that Chi6769 was capable of hydrolyzing chitin to $(GlcNAc)_3$, $(GlcNAc)_2$ and GlcNAc. $(GlcNAc)_2$ was the main hydrolyzate. The antifungal activity result showed that Chi6769 exhibited strong antifungal activity toward *Valsa mali* 03–8. Overall, Chi6769 was potential to be a novel biofunctional chitinase that could be used as a biological agent in the control of plant diseases.

1. Introduction

Chitinases, vary in size from 20 kDa to 90 kDa [3], are glycosyl hydrolases that hydrolyze chitin. According to their cleavage site, chitinases can be classified as endo- or exo-chitinases. Endo-chitinases cleave β-1, 4-glucosidic linkages in chitin [25] to form diacetyl chitobiose and soluble low molecular weight chitooligosaccharides, such as chitotriose and chitosan tetrasaccharide [19]. There are two types of exo-chitinases: one is β-1,4-glucosidase, which can decompose the oligomers produced by chitinase to *N*-acetylglucosamine [10]; the other type is chitin hydrolases that catalyze the release of diacetyl chitobiose from non-reducing ends. Under normal circumstances chitin endonuclease and exonuclease are active at the same time.

Most plant diseases are caused by pathogenic fungi, and chitin is an important cell wall component of most pathogenic fungi. Chitinase-producing microorganisms can break down chitin, which can be used as biological controls of fungal infection in the crops [6]. *Aspergillus flavus* toxin can be cleaved by *Bacillus circulans*, and with an increase in the concentration of chitinase, the cleavage efficiency increased [11]. The plant growth-promoting rhizobacteria *Pseudomonas fluorescens* can produce extracellular chitinases to inhibit mycelial growth, and have been used to treat fungal diseases. The possible mechanism of fungal growth inhibition is via the hydrolysis of chitin chains at the tip of mycelium by chitinase, causing a thinner cell wall and rupturing the protoplast. At the same time, newly synthesized chitin can also be degraded by chitinase, leading to inhibition of elongation and mycelial growth. It was found that chitinase can specifically inhibit fungal spore germination. *Trichoderma harzianum* was widely used in the biological control of plant diseases, as it can secrete cell degrading enzymes, in particular chitinase, which plays an important role in the control of pathogens.

Strain Hhs.015 was isolated from the roots of cucumber, and classified into the genus *Saccharothrix* based on its 16S rDNA sequence. Based on the result of polyphasic taxonomy, the strain was identified as a novel species named *Saccharothrix yanglingensis* [27]. The genus *Saccharothrix* was first isolated from Australian soil samples [13]. In addition, *Saccharothrix* species were found in the following gold mines [15], plant litter [18] and soils [17]. Many of these strains produce secondary metabolites which are active against phytopathogenic microorganisms, toxigenic fungi, and drug-resistant pathogens [4,21]. Our previous results indicated that Hhs.015 had good inhibitory activity on the apple tree canker pathogen, *Valsa mali,* and other pathogens. Further study showed that chitinase can effectively degrade chitin, an important component in the cell wall of pathogenic fungi, and inhibit the growth of the pathogen. Therefore, the chitinase of strain Hhs.015 was studied to determine whether chitinase was involved in the biocontrol reaction.

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2. Materials and methods

2.1. Materials

Chitin (from crab shells), *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (*p*-NP-GlcNAc) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-Ascorbic acid/Vitamin C (Vc); Thiamine/Vitamin B1 (V_{B1}); Lysozyme; N-acetyl chitooligosaccharides (COSs) with degrees of polymerization (DP) from 2 to 3 were purchased from Solarbio (Beijing, China). HisTrap HP was obtained from GE Healthcare (Piscataway, NJ, USA). Silica gel plates were produced by Haiyang Co. (Qingdao, China). Gause I Medium: Soluble starch 20.0 g/L, KNO_3 1.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, agar 20.0 g/L, distilled water 1.0 L, pH 7.4–7.6. TSB liquid medium: Peptone 15.0 g/L, Soya peptone 5.0 g/L, NaCl 5.0 g/L, distilled water 1.0 L, pH 7.0–7.4. All other chemicals used were of analytical grade unless otherwise specified.

2.2. Strain and culture conditions

Isolate of *S. yanglingensis* Hhs.015 was provided by the Laboratory of Integrated Management of Plant Diseases, College of Plant Protection, Northwest A & F University, Yangling, Shaanxi Province, China. It was cultured on Gause's No 1 synthetic agar medium plates at 28 °C for 7 d.

2.3. Cloning of chitinase gene and its sequence analysis

A single colony of Hhs.015 was inoculated on Gause I medium plate and incubated at 28 °C for 72 h, then transferred in TSB liquid medium and incubated for 72 h at 28 °C and 150 rpm. The cells were collected by centrifugation, added lysozyme (10 mg/mL) in tubes and incubated at 37 °C and 200 rpm for overnight, and DNA was extracted with CTAB method. Finally the DNA was resuspended in sterile ddH_2O and stored at −20 °C until use. Primers (6769F and 6769R) were designed according to genome information of strain Hhs.015. The partial core region of the chitinase gene was amplified using the degenerate primers, 6769F (5′-GGAATTCCATATGCCGCGGTCCTCACCCT-3′) and 6769R (5′-CCGCTCGAGCGAGCGGGATCGTCCGCTT-3′). The PCR conditions were as follows: 60 s at 95 °C, followed by 35 cycles of 95 °C for 15 s, 60°Cfor 15 s, and 72°Cfor 90 s, followed by a final extension 72 °C for 5 min.

Predicted protein sequences were compared to each database (e value was $1e^{-10}$). COG, GO, KEGG, and NR were used to classify each protein and obtain gene function information. The basic physical and chemical properties of the chitinase were analyzed using the online software tools, Protparam (http://web.expasy.org/protparam) and ProtScale (http://web.expasy.org/protscale/). The prediction of transmembrane structure of the chitinase was performed using the online software tools ProtScale and TMHMM. The secondary structure of the chitinase was predicted by SCRATCH Protein Predictor and PredictProtein. Signal peptide analysis and cleavage site prediction were performed using the online tool SignalP (http://www.cbs.dtu.dk/services/SignalP/). NCBI conserved domain databases were used for domain prediction. The online software tool SWISS-MODEL was used to predict the tertiary structure of the chitinase. Chitinase amino acid sequence homology was analyzed by DNAMAN software and the phylogenetic analysis was performed using the NJ method implemented in MEGA6.0 software.

2.4. Expression of the chitinase gene (Chi6769) in E. coli BL21

PCR products were purified, digested with the restriction enzymes *Nde*I and *Xho*I and then ligated into the vector pET28a, which has been digested with the same enzymes. The recombinant vector was transformed into *E. coli* BL21 (DE3) and the positive clones were cultured in LB medium (containing 50 μg/mL kanamycin) at 37 °C and 150 rpm. When the optical density (OD_{600}) of the culture medium reached 0.5, isopropyl-β-D-thio-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was incubated for 9 h at 16 °C.

2.5. Purification of the recombinant chitinase Chi6769

Culture broth was centrifugated at 8000 rpm for 10 min. The pellet was collected, re-suspended in 50 mM PBS buffer (pH 7.0), and ultrasonicated. Cell debris was removed by centrifugation at 8000 rpm for 10 min, and the supernatant was used as crude extract. After dialyzing against 0.1 mM PBS buffer at pH 7.0 overnight, 5 mL crude extract was loaded on a HisTrap HP column (Volume 1 mL) pre-equilibrated with binding buffer (50 mM PBS buffer at pH 7.0 containing 40 mM imidazole and 4 M NaCl). After binding for 20 min, weakly bound impurities were removed with 10 times column volumes of binding buffer, followed by elution buffer C (50 mM PBS buffer at pH 7.0 containing 4 M imidazole and 2 M NaCl). The eluted fractions were collected and the purity was determined by Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE). All purification steps were performed at 4 °C with flow rate of 1 mL/min, unless otherwise stated. SDS–PAGE was carried out as described by Laemmli using 8% separating gel and 5% stacking gel [14]. The protein samples were mixed with gel loading buffer before loading. Protein bands were stained with Coomassie Brilliant Blue G-250. The protein marker was Rainbow marker (Solarbio). Zymograms were performed as previously described [26].

2.6. Chitinase assay and protein determination

p-NP-GlcNAc was dissolved in 50 mM sodium phosphate buffer (PBS, pH 7.0) to a final concentration of 0.25 mM 1.95 mL of the solution was mixed with 50 μL enzyme solution and incubated at 37 °C for 10 min, followed by the addition of 2 mL 0.5 M NaOH to terminate the reaction. The release of *p*-nitrophenol from *p*-NP-GlcNAc was measured by recording the absorbance at 410 nm and then compared to the absorbance measurements of *p*-nitrophenol standards (20–100 μM). One unit of chitinase activity was defined as the amount of enzyme needed to release 1 μmol *p*-nitrophenol from the substrate per minute at above conditions. Chitinase activity was determined in triplicate experiments. The protein concentration was determined by Bradford method [5] using bovine serum albumin as standard.

2.7. Characterization of the purified Chi6769

The effect of pH (from 2.0 to 11.0) on enzyme activity was determined using the method described above. The pH stability was measured using purified chitinase in a solution without substrate at pH 3.0 to 9.0, and was determined as the percentage of residual enzyme.

Optimal temperature assays were also performed. The reaction mixture was incubated over a temperature range of 22–61 °C at intervals of 3 °C to find out the effect of temperature on the activity of the purified enzyme. The reaction was incubated at the chosen temperature for 30 min prior to the addition of substrate and the percentage of residual enzyme activity was calculated. Thermal inactivation of the enzyme was evaluated by incubating the enzyme in 50 mM PBS buffer (pH 7.0) at 50 °C for 3 h. Aliquots were withdrawn at different time intervals and their enzyme activities were measured according to the standard assay.

Effects of metal ions on enzyme activity were investigated by adding Fe^{3+} ($FeNO_3$), Mg^{2+} ($MgSO_4$), Mn^{2+} ($MnSO_4$), Cu^{2+} ($CuSO_4$), Zn^{2+} ($ZnSO_4$), Fe^{2+} ($FeCl_2$), K^+ (KCl), Ca^{2+}($CaCl_2$), Cr^{3+}($Cr_2(SO_4)_3$) or Na^+ (NaCl) into the reaction systems at the final concentration of 10 mM.

Chemical reagents, Vc, V_{B1}, coenzyme, soluble starch, glutamine, β-cyclodextrin, galactose, EGTA, EDTA, L-rhamnose,L-asparagine, L-proline and sorbitol were also investigated at a concentration of 10 mM for their impact on enzyme stability. Chitinase activity was measured using the method described above. The Michaelis-Menten constant (*K*m) and maximum velocity (*V*max) of the chitinase samples were calculated