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Short communication

Expression patterns of chitinase and chitosanase produced from *Bacillus cereus* in suppression of phytopathogen

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ABSTRACT

Bacillus cereus MP-310 was incubated on various culture media substrates as LB, colloidal chitin, chitosan powder, and chitosan beads to investigate the concurrent expression patterns of chitinase and chitosanase isozymes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Chitinase activity increased rapidly with a maximum level after 6 days of incubation in CM-chitin medium. Major bands of chitinase isozymes were strongly expressed on SDS-PAGE in LB medium (four bands) and in colloidal chitin medium (five bands) after 6 days after incubation, and in chitosan powder medium (one band) and in chitosan beads medium (five bands) after 12 days after incubation. A major band of chitosanase isozymes was strongly expressed on SDS-PAGE in chitosan powder medium (one band) and in chitosan beads medium (one band) after 12 days of incubation.

1. Introduction

Chitin, a partly deacetylated (1-4)-2-acetamido-2-deoxy- β-D-glucan, is one of the most widespread principal structural polymers in nature [1]. Chitinases (Chi I and Chi II) from *Bacillus cereus* TKU027 produce chitin oligosaccharides from shrimp chitin and chitoligosaccharides from chitosan with 60% deacetylation [2]. Endophytic *B. cereus* strain 65 isolated from *Sinapis* produces chitinase, which is classified as a chitobiosidase [3].

Microbial chitosanases have been studied mainly as inducible enzymes by colloidal chitosan [4,5]. Additionally, bacterial chitosanase is induced by various substrates such as chitosan powder in *Paenibacillus* sp. [6], chitin in *Streptomyces roseolus* [7], colloidal chitin in *B. cereus* [8], squid pen powder in *Acinetobacter calcoaceticus* [9], shrimp head powder in *B. cereus* [2], and shrimp shell powder in *Serratia marcescens* [10].

Chitinases and chitosanase produced from *Bacillus* sp. with antagonistic activity are capable of inhibiting fungal growth such as *Fusarium oxysporum f.* sp. *meloni*, *Rhizoctonia solani* [3], and *R. solani* [4].

The characteristics of chitinase produced from *B. cereus* have been reported. However, concurrent expression of chitinase and chitosanase from *B. cereus* has not been extensively studied. We found the chitinase and chitosanase from *B. cereus* are produced on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after an incubation in various substrate media.

In this study *B. cereus* was cultured on three different culture media (LB, chitosan powder, and chitosan beads medium), and then compared with three media for the production of chitinase and chitosanase isozymes on SDS-PAGE including glycol chitin and glycol chitosan, respectively. The objectives of this study were to investigate the properties of chitinase isozymes from *B. cereus*, and to investigate the concurrent expression patterns of chitinase and chitosanase isozymes on SDS-PAGE with chitosan substrates.

2. Materials and methods

2.1. Isolation and identification of chitinolytic bacterium

The cast-off shells of cicadas were collected from the arboretum of Chonnam National University, Korea. The sample was grained and inoculated on 0.5% colloidal chitin (pH 6.0) agar medium at 30 °C for 3 days. One bacterium having strong chitinolytic activity was isolated and selected for further characterization. Chitin and chitosan (deacetylation 92%) were obtained from Keumho Chemical Co., Ltd. (Seoul, Korea).

Polymerase chain reaction (PCR), (GeneAmp 9700, Applied Biosystems, Foster City, CA, USA) was performed to amplify a part of the 16S rRNA gene to identify the bacterium. The primers used were the forward primer 9F (5′-GAG TTT GAT CCT GGC TCA G-3′) and the

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reverse primer 1412R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The nucleotide sequence of the 16S rRNA gene from MP-306 was determined by an ABI PRISM Big Dye™ Terminator Cycle Sequencing Kits (Applied Biosystems) and ABI PRISM 3730xl Analyzer (Applied Biosystems) by Geno Tech Co. (Daejeon, Korea). The nucleotide sequence of the 16S rRNA gene of the MP-310 was compared with published 16S rRNA sequences using a Blast search at NCBI [11].

2.2. Preparation of chitin and chitosan substrates

Red crab shell was obtained from a seafood market and cleaned. Crab shell colloidal chitin was prepared by the method of Berger and Reynolds [12]. The chitin-protein complex from crab shell was prepared by stirring in 5% hydrochloric acid solution at room temperature for 3 h to remove the calcium carbonate. The *N*-carboxymethyl-chitin (CM-chitin) was prepared from the crab shell by modifying a carboxymethylation procedure [13]. Chitin powder (5 g, 60 mesh) was suspended in 20 mL of 60% NaOH solution including 0.1% (20 mg) SDS at 4 °C, and the slurry was kept at −20 °C overnight after standing for 1 h at 4 °C [14]. The frozen alkali-chitin was suspended in 150 mL isopropyl alcohol for 1 h at 30 °C, and monochloroacetic acid ($CH_2ClCOOH$) was added in portions with mechanical stirring until the reaction mixture was neutralized. The product was washed with 600 mL 70% and 80% methanol, and then with 100% methanol. The powder was dried at 60 °C for 3 h. The culture media used in this study were prepared to investigate the activity of chitinase.

Chitosan beads were prepared using chitosan powder (7 g) dissolved in 100 mL of 7% acetic acid. The resulting viscous solution was de-gassed under vacuum, and dropped into 200 mL of an alkali coagulating solution (H_2O:MeOH:NaOH = 4:5:1, wt/wt) to prepare highly swollen beads. The beads were collected and thoroughly washed with distilled water [15]. The chitosan beads were dried at 60 °C as 1–2 mm average diameter beads and used for further enzyme activity.

2.3. Mycelial growth inhibition

R. solani KACC 40111, *Fusarium solani* KACC 40384, *F. oxysporum* KACC 40032, and *Botrytis cinerea* KACC 40574 were grown in potato dextrose agar (PDA) to investigate inhibition of mycelial growth with strain MP-310. The plate was incubated at 26 °C for 3 days, and the distances between the edges of the MP-310 and growth of fungal mycelium were observed measured.

2.4. Determination of chitinase activity

B. cereus MP-310 was incubated on 0.5% crab shell, 0.5% chitin protein complex, and 0.5% CM-chitin medium at 30 °C for 6 days. Chitinase assay mixture consisted of 50 μL of sample, 500 μL of 0.5% colloidal chitin, and 450 μL of 50 mM sodium acetate buffer (pH 5.0) [16]. Following incubation at 37 °C for 1 h, 200 μL of 1 N NaOH was added. Next, the sample was briefly centrifuged (10,000× *g*, 5 min), after which 500 μL of the supernatant was mixed with 1 mL of Scales' reagent and then heated in boiling water for 15 min. The absorbance was measured immediately at 420 nm using a spectrophotometer (Mecasys, Optizen 3220UV, Seoul, Korea). The quantity of reducing sugars was calculated based on comparison with a standard curve generated from known concentrations of GlcNAc (0–100 μg). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour. The protein concentration was determined using the method described by Bradford [17].

2.5. Activity staining of chitinase and chitosanase

To investigate the consecutive expression patterns of chitinase, *B. cereus* MP-310 was incubated on LB medium and 0.5% colloidal chitin at 30 °C for 6 days. The daily supernatant of two culture media was loaded on SDS-PAGE gels to investigate the expression patterns of chitinase isozymes, according to the method described by Laemmli [18].

B. cereus MP-310 was incubated on LB medium at 30 °C for 2–3 days, and on 0.5% chitosan powder and 0.5% chitosan beads at 30 °C for 12 days to comparatively analyze the two enzymes. The supernatant of three culture media were loaded on SDS-PAGE gels to investigate the expression patterns of chitinase and chitosanase isozymes.

Electrophoresis was accomplished using a Bio-Rad Mini-PROTEAN (80 × 73 × 1.5 mm) machine. The 12% SDS-PAGE gel was stained with 0.12% Coomassie Brilliant Blue R-250 staining. To evaluate the active staining of chitosanase and chitinase, 12% SDS-PAGE containing 0.01% glycol chitosan and 0.01% glycol chitin was conducted according to the method described by Trudel & Asselin [19], respectively. The gel was incubated in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% skim milk at 37 °C for 2 h with reciprocal shaking. A subsequent incubation was then conducted overnight under the same conditions, but without skim milk in the buffer solution. The gel was then immersed in 500 mM Tris–HCl (pH 8.9) solution containing 0.01% Calcofluor white M2R (Sigma F3397; St. Louis, MO, USA). The lysed zones were visualized and photographed using a UV transilluminator (Daihan Sci. Co., WGD-30, Seoul, Korea).

3. Results and discussion

3.1. Identification of chitinolytic and chitosanolytic bacterium

The MP-310 isolate had strong chitinolytic and chitosanolytic activity on agar medium containing 0.5% (w/v) colloidal chitin and 0.5% (w/v) colloidal chitosan, showing clear zone around colonies after a 2 day incubation at 30 °C (Fig. 1A). The bacterium was identified as *B. cereus* and named as *B. cereus* MP-310 based on the nucleotide sequence of a conserved segment of 16S rRNA gene. Only a two base difference was observed between MP-310 (ID JN816403) and the *B. cereus* DL-3-1 16S rRNA sequence (ID GQ199590) within the 1446 bp sequence. MP-310 culture broth had strong antifungal activity on PDA medium for the phytopathogens *R. solani*, *F. solani*, *F. oxysporum*, and *B. cinerea* (Fig. 1B–E). Crude protein produced from *B. cereus* strain 65 showed inhibited the spore germination rate of *F. oxysporum f.* sp. *meloni* [3]. Applying *B. cereus* strain 65 to soil resulted in a protective effect against *R. solani* in cotton seeds. Purified chitosanase from *B. cereus* D-11 showed antifungal activity against *R. solani* AG-1 on a plate [4].

3.2. Cell growth, protein content, and chitinase activity

Cell growth and protein contents of *B. cereus* MP-310 were determined in 0.5% crab shell, 0.5% chitin protein complex, and 0.5% CM-chitin medium to investigate the a basic properties of *B. cereus* MP-310 for expression patterns of enzyme (Fig. 2A and B). Additionally, chitinase activity of *B. cereus* MP-310 was determined in 0.5% crab shell, 0.5% chitin protein complex, and 0.5% CM-chitin medium (Fig. 3). Cell growth increased rapidly for 2 days and then was maintained continuously in crab shell and chitin protein complex medium (Fig. 2A). Protein content increased for 4 days in the same medium (40.9 μg/mL in crab shell and 81.8 μg/mL in chitin protein complex) and then was maintained continuously during the incubation (Fig. 2B). *B. cereus* MP-310 cell growth increased

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