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Purification and characterization of chitosanase from *Bacillus cereus* D-11

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Abstract

A chitosanase-producing bacterium was isolated from Taiwan soils and identified as *Bacillus cereus* D-11 based on the biochemical properties and 16S rRNA gene sequence. The optimal medium for enzyme production consisted of 0.7% colloidal chitosan, 1% yeast extract, and 1% NaCl at an initial pH of 7.0 with 0.5% inoculation concentration (1.4×10^8 CFU/ml). After cultivation at 30 °C for 3 days, the maximal activity of 4.85 U/ml was observed. An extracellular chitosanase produced from *B. cereus* D-11 was concentrated by lyophilization and purified by Sephadex G-150 gel filtration and CM-Sephadex ion exchange column chromatography. The molecular weight of the purified chitosanase was estimated to be 41 kDa by 12.5% SDS–PAGE. The optimal pH and temperature for the chitosanase were 6.0 and 60 °C, respectively. The enzyme was stable below 50 °C and from pH 5 to 10. N-terminal amino acid sequence exhibited highest homology to the chitosanases belonging to glycoside hydrolase family 8. The enzyme was inhibited by 10 mM 2-hydroxy-5-nitrobenzyl bromide but activated by phenylglyoxal and chloramine T. The K_m and V_{max} values were 7.5 mg/ml and 2.15×10^{-7} mol/mg/s for soluble chitosan (degree of deacetylation, DD 86%) as substrate. The D-11 chitosanase degraded chitosan with DD ranging from 70% to 100%, but did not degrade chitin. The most susceptible substrate was 86% deacetylated chitosan. Furthermore, the D-11 chitosanase inhibited the mycelial growth of *Rhizoctonia solani* on PDA medium.

Keywords: *Bacillus cereus* D-11; Chitosanase; Antifungal activity; Glycoside hydrolase family 8; Degree of deacetylation

1. Introduction

Chitosan is a linear polysaccharide composed of β-(1→4)-linked D-glucosamine residues. In nature, the polymer is partially acetylated and, in fact, the name chitosan describes a wide range of polymers with various proportions of D-glucosamine and *N*-acetyl-D-glucosamine residues (Pelletier & Sygusch, 1990).

Previously, several microorganisms including bacteria were reported to efficiently produce chitosanases (EC 3.2.1.132) to degrade chitosan to glucosamine oligomers (Jo et al., 2003; Kim, Kang, Chung, Kim, & Chung, 2004). Furthermore, fungi (Chen, Xia, & Yu, 2005; Zhang et al., 2000) and actinomycetes (Okajima, Ando, Shinoyama, & Fujii, 1994; Shimosaka, Nogawa, Wang, Kumehara, & Okazaki, 1995) also produce chitosan oligomers from chitosan. These chitosanases belong to various GH (glycosyl hydrolase) families including GH-5, GH-8, GH-46, GH-75, and GH-80, according to their amino acid sequences (Jung, Kuk, Kim, Park, & Park, 2005). The characteristics of the chitosanases produced from *Bacillus cereus* P16 (Jo et al., 2003), *Bacillus* sp. GM44 (Choi, Kim, Piao, Yun, & Shin, 2004), *Bacillus* sp. KSM-330 (Ozaki, Sumitomo, & Ito, 1991), *Bacillus circulans* WL-12 (Mitsutomi et al., 1998), and *Bacillus* sp. 7-M (Uchida & Ohtakara, 1988) have been well investigated and belong to the GH-8.

Chitosanases from individual organisms differ in their pattern of hydrolytic activity. Most microbial chitosanases

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catalyze an endo-type cleavage reaction in which the relative velocity is highly dependent on the deacetylation of chitosan (Shimosaka et al., 1995). Recently, much attention has been paid to converting chitosan to safe and functional chitooligosaccharides, because chitooligosaccharides show strong physiological activities, such as antitumor effects (Maeda & Kimura, 2004) and antimicrobial activity (Wang et al., 2007). Application of novel and potential chitosanases from chitosanase-producing microorganisms has recently been one of the advanced and alternative approaches for producing chitosan oligomers.

The purpose of this study was to isolate chitosanase-producing bacteria, then to purify and characterize the chitosanase from *B. cereus* D-11, which has high chitosanolytic potential.

2. Materials and methods

2.1. Materials

Chitosans with various deacetylation degrees were purchased from Taehoon Bio Ltd. (Seoul, Korea). The chitosan used in this report was 86% deacetylated unless otherwise stated. Chitosan oligomers $(GlcN)_{1-7}$ were purchased from Wako Chemicals (Osaka, Japan). Sephadex G-150 and CM-Sephadex were purchased from Pharmacia and Sigma, respectively. Buffer solutions and other chemicals were reagent grade. *Rhizoctonia solani* AG-1 (B) Khun (KACC 40111) was obtained from Korea Agricultural Culture Collection (KACC).

2.2. Isolation and identification of chitosanolytic bacterium

One bacterium D-11 was screened from Taiwan soil samples. Serially diluted soil samples were inoculated on plates containing 1% NaCl, 1% tryptone, 0.5% colloidal chitosan (basic medium) and 2% agar and incubated at 30 °C for 3 days (Jo et al., 2003). A single colony showing prominent chitosanolytic activity was selected and subjected to taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology* (Sneath, Mair, Sharpe, & Holt, 1986). To further identify the bacterium, polymerase chain reaction (PCR) was performed to amplify part of the bacterial 16S rRNA gene. The forward and reverse primers were 5′-ACGGCTACCTTGTTACGACT-3′ and 5′-CCC ACTGCCTCCCGTAAGGAGT-3′, respectively. The PCR product was cloned using pGEM-T Easy vector (Promega, USA). The nucleotide sequence of the 16S rRNA gene of D-11 was determined by an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, USA) and compared with published 16S rRNA sequences in a NCBI BLAST search.

2.3. Purification of chitosanase

D-11 was cultured in a medium containing 0.5% colloidal chitosan, 1% NaCl, and 1% tryptone (basic medium) at 30 °C for 3 days. The crude enzyme after concentration was lyophilized, dissolved in an appropriate volume of 50 mM sodium acetate buffer at pH 6.0, and then dialyzed. The solution was loaded onto a Sephadex G-150 gel filtration column (2.5 × 40 cm) which was equilibrated with the same buffer. The active fractions were further purified on a CM-Sephadex column (2.5 × 40 cm). The proteins were eluted at a flow rate of 0.9 ml/min, and 4-ml fractions were collected. The protein profile was monitored by the absorbance at 280 nm. Chitosanase activity was assayed using soluble chitosan (DD 86%) as a substrate (Imoto & Yagishita, 1971). Each reaction mixture contained 0.9 ml of 1% soluble chitosan, 80 μl of 100 mM sodium acetate buffer (pH 6), and 20 μl of enzyme solution. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 200 μl of 1 M NaOH. The amount of reducing sugar was determined by modification of Schale's method (Imoto & Yagishita, 1971) or the dinitrosalicylic acid method (Miller, 1959) with glucosamine as a standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute. Protein concentration was measured by Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

2.4. Characterization of the purified chitosanase

To determine optimum temperature, reaction mixtures were incubated at various temperatures for 30 min in the acetate buffer, pH 6.0, as described by Jo et al., 2003. To determine temperature stability, the residual activity was measured after pre-incubation of the enzyme at various temperatures for 1 h at pH 6.0 in the absence of substrate. For determination of the optimum pH, enzyme solutions were incubated with soluble chitosan in various pH buffers at 37 °C for 30 min. Mcllvaine buffer (pH 3.5), sodium acetate buffer (pH 4–6), Tris–HCl buffer (pH 7–8), and carbonate buffer (pH 9–11) were used for pH adjusting. For pH stability, the enzyme solutions were preincubated for 1 h at 4 °C in various pH buffers (50 mM). After adjusting to pH 6.0 with the acetate buffer, the enzyme solutions were incubated with substrate at 37 °C for 30 min. For substrate specificity, enzyme activity was determined toward several chitosan-derived and non-chitosan substrates (0.5% w v^{-1}). For metal ion effect, the chitosanase was pre-incubated with 10 mM metal ions, such as Mg^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, and Hg^{2+}. For kinetic constants, 20 μl of enzyme (4.2 U/ml) was incubated with soluble chitosan at concentrations between 0.5 and 2.5 mg/ml at 37 °C for 1 h. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined by Lineweaver–Burk transformation (Price & Storck, 1975).

2.5. Modification of amino acid residues

The amino acid residues or functional groups were modified by incubating the purified enzyme (2.0 mU) with several modifying reagents at 25 °C in the respective buffers