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Purification and properties of a chitinase from *Penicillium* sp. LYG 0704

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ABSTRACT

The chitinase producing *Penicillium* sp. LYG 0704 was procured from soil of the Chonnam National University crop field. The chitinase activity was detected after the first day which increased gradually and reached its maximum after 3 days of cultivation. The chitinase was purified from a culture medium by precipitation with isopropanol and column chromatography with Mono Q and Butyl-Sepharose. The molecular mass of chitinase was estimated to be 47 kDa by SDS–PAGE. Optimal pH and temperature were 5.0 and 40 °C, respectively. The N-terminal amino acid sequence of the enzyme was determined to be ^{1}AGSYRSVAYFVDWAI^{15}. The fully cloned gene, 1287 bp in size, encoded a single peptide of 429 amino acids. BLAST search of the chitinase gene sequence showed similarity with chitinase of *Aspergillus fumigatus* Af293 chitinase gene (58%) and *A. fumigatus* class V chitinase *ChiB1* gene (56%).

After cellulose, chitin is the most abundant source of organic compound in nature. Chitin is a long-chain polymer of *N*-acetylglucosamine (GlcNAc)[1] units. These units form covalent β-1,4 linkages similar to those between glucose units forming cellulose. Chitin is the major structural component of fungal cell walls, arthropod exoskeletons, and crustacean shells [1].

Chitooligosaccharides, which are amino sugars, are known to possess various biological roles, including as antitumor agents and elicitors [2]. The role of GlcNAc as a therapeutic agent in the treatment of osteoarthritis has also received considerable attention [3]. GlcNAc has historically been produced by acid hydrolysis of chitin with concentrated HCl at high temperature (above 80 °C) [4]. However, this chemical process has problems including the production of acidic wastes, low yield, and high cost. As a result, the focus has shifted to enzymatic hydrolysis as a more appropriate method of GlcNAc production from chitin [5,6].

Chitin hydrolyzing enzymes are classified into three categories based on the manner in which they cleave chitin chains. These are endochitinases, exochitinases, and *N*-acetylglucosaminases. Endochitinases randomly cleave β-1,4-glycosidic bonds of chitin, whereas exochitinases cleave the chain from the nonreducing end to form diacetyl-chitobiose $(GlcNAc)_2$. *N*-Acetylglucosaminases hydrolyze $GlcNAc_2$ into GlcNAc or produce GlcNAc from the nonreducing end of *N*-acetyl-chitooligosaccharides [7].

Based on amino acid sequence similarity of their catalytic domains, chitinases are classified into families 18 and 19 of glycosyl hydrolases [8]. Family 18 chitinases are distributed widely in bacteria, fungi, viruses, animals, and some plant species, while family 19 chitinases are found only in higher plants, but were recently identified in *Streptomyces griseus* [9]. The chitinases of these two families differ in their amino acid sequences, three-dimensional (3D) structures [10,11], and molecular mechanisms of catalytic reactions. Therefore, they are likely to have evolved from different ancestors and followed separate evolutionary pathways. In this study, we describe the cloning, purification, and characterization of chitinase from *Penicillium* sp. LYG 0704 and cloning of the encoding gene.

Materials and methods

Strains and plasmids

For the purpose of cloning, transformation, and propagation of recombinant chitinase gene plasmid, *Escherichia coli* TOP10F' [{F- mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ (ara-leu) 7697 galU galK rpsL(StrR) endA1 λ-}, Invitrogen, USA] was used as a host strain. For TA cloning, Plasmid pGEM-T Easy Vector (Promega, USA), which contains the linearized single overhanging 3′ thymine (T) residues, was used.

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[1] *Abbreviations used:* GlcNAc, *N*-acetylglucosamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM, scanning electron microscopy.

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Microorganisms and culture conditions

A fungal strain exhibiting strong extracellular chitinase activity was isolated from soil using chitin–agar plate mixtures containing 1% colloidal chitin, 0.42% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.02% urea, 0.03% $CaCl_2$, 0.03% $MgSO_4{\cdot}7H_2O$, 0.1% proteose peptone, 0.2% Tween 80, 0.2% trace element solution (0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $MnSO_4{\cdot}H_2O$, 0.14% $ZnSO_4{\cdot}7H_2O$, 0.2% $CoCl_2$), and 1.7% agar at pH 7.0. The isolated LYG 0704 was cultured in 500 ml chitin medium for 3 days at 30 °C on a rotary shaker with a rotation speed of 150 rpm.

Fungal isolation

Identification of the isolated LYG 0704, 18S rDNA, ITS1 DNA, 5.8S rDNA, and ITS2 DNA was carried out for DNA sequence analysis. Genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega Inc., Madison, WI, USA) following the manufacturer's method. The first PCR amplified the region between 18S rDNA and ITS2. It was noticed that the first PCR primers were universal primer EF4 (5′-GGAAGGGGTGTATTTATTAG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The second PCR amplified the full length of 18S rDNA. These second PCR primers were identified as universal primer EF4 and EF3r (5′-TCCTCTAAATGACCAAGTTTG-3′). Reagents added to a PCR tube for amplification contained the following mixtures: 2 μl of genomic DNA, 4 μl of 5× PCR buffer, 1 μl EF4 primer, 1 μl ITS4 primer (or EF3r primer), 0.8 μl of 2.5 mM dNTP, and 0.2 μl of Taq DNA polymerase (5 U/μl). PCR amplification was conducted according to the following thermal cycle: 1 cycle of 94 °C for 3 min: 30 cycles of 94 °C for 20 s, 48 °C for 20 s, 72 °C for 2 min: final extension for 5 min at 72 °C [12,13] after which PCR production was purified (JETsorb DNA Extraction Kit). Transformation amplified DNA to *E. coli* Top10 after ligation to pGEM-T easy vector (Promega), after which the DNA was isolated using Wizard® PlusSV Minipreps DNA Purification Kit (Promega Co., USA). Plasmid DNA sequencing was conducted at the KBSI (Korea Basic Science Institute, Gwangju, Korea) using a Model ABI PRISM 377 (Perkin Elmer, USA) DNA sequencer. The sequence of 18S rDNA and 5.8S rDNA was obtained and compared with the NCBI database using a BLAST search.

Table 1
Purification of chitinase from *Penicillium* sp. LYG 0704.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtered	396	306	0.7	100	1
Isopropanol precipitation	114	134	1.1	43.8	1.6
Mono Q	7.3	65.7	9	21.4	12.8
Butyl-Sepharose	2.2	27.1	12.3	8.8	17.6

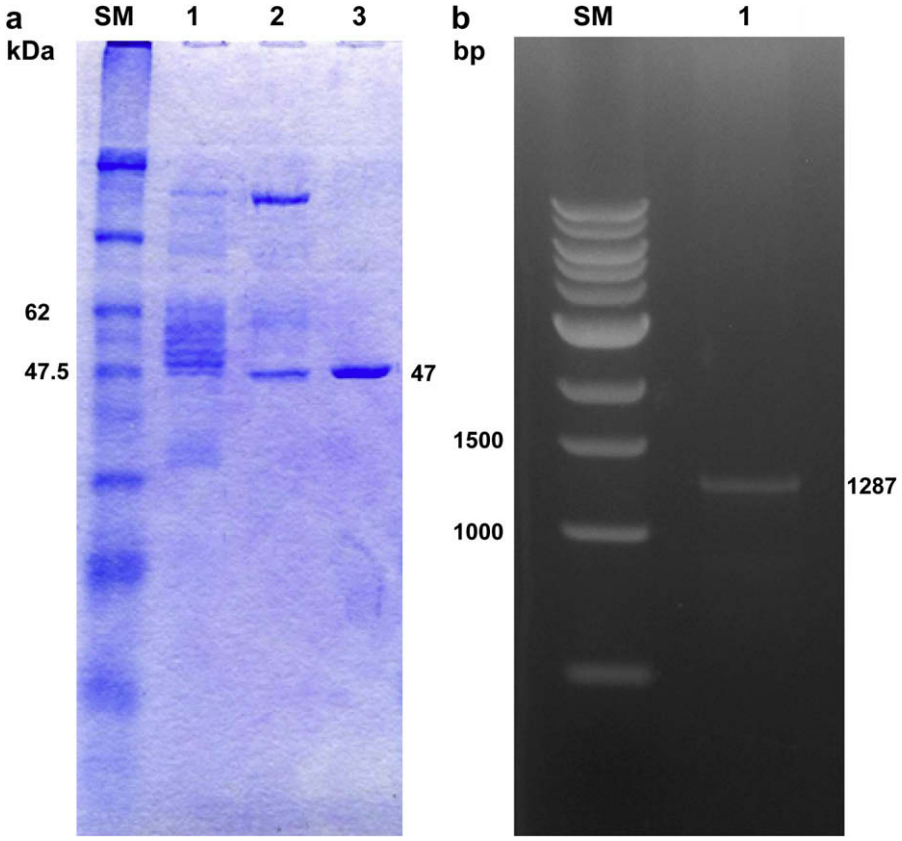

Fig. 1. Homogeneity of purified chitinase and synthesis of chitinase genes. (a) Proteins containing chitinase fractions from each purification step were detected by 12% SDS–PAGE. SM, molecular marker (Promega); lane 2, culture filtrate; lane 3, Mono Q chromatography; lane 4, Butyl-Sepharose chromatography. (b) Analysis of amplified DNA on 0.8% agarose gel. SM, 1 kb DNA ladder (Bioneer, Korea); lane 1, full length chitinase gene.