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NOTE

Enhanced Expression of Chitinase during the Autolysis of Mushroom in *Coprinellus congregatus*

Hyangsoon Lim and Hyoung T. Choi*

Molecular Microbiology Lab, Department of Biochemistry, Kangwon National University, Chuncheon 200-701, Republic of Korea

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Fungal cell walls consist of various glucans and chitin. An inky cap, *Coprinellus congregatus*, produced mushrooms at 25°C in a regime of 15 h light/9 h dark, and then the mushroom was autolyzed rapidly to generate black liquid droplets where no cell wall was detected by microscopy. A chitinase cDNA from the matured mushroom cells of *C. congregatus* that consisted of 1,541 nucleotides was successfully cloned using the rapid amplification of cDNA ends (RACE)-PCR technique. Its deduced 441 amino acid sequence had the conserved catalytic domain as in other fungal chitinase family 18. Chitinase activity was higher at the matured mushroom stage than primordial and young mushroom stage. When the expression of the cloned chitinase was examined by real-time PCR using the chitinase-specific primers, it was increased more than twice to 20 times during the autolytic process of mushroom than young mushroom or primordial stages, respectively.

***Keywords*:** chitinase, gene expression, inky cap

The cell wall of filamentous fungi consists of diverse polymers such as α- and β-glucans, other sugar polymers and chitins. Chitin, a polymer of N-acetylglucosamine is also found in many diverse organisms such as insects, crustaceans as well as fungi. Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glycosidic linkages of the N-acetylglucosamine polymer chitin. Chitinases can be grouped in two families, family 18 and family 19 of glycoside hydrolases by their amino acid sequences (Henrissat, 1999). Chitinases in family 19 include most plant chitinases and *Streptomyces* chitinases, whereas fungal chitinases belong to family 18 (Henrissat, 1999). Most filamentous fungi require chitinase during growth phase to enlarge surface area of their cell wall. ChiA, a glycosylphosphatidylinositol-anchored chitinase is localized at polarized growth site in *Aspergillus nidulans* (Yamazaki *et al*., 2008). When opportunistically pathogenic fungi infected human body, their cell morphologies showed dimorphic changes to protect themselves from the host defense mechanism and to disseminate their own progenies inside the host (Sundstrom, 2003). *Candida albicans* showed different chitin content during its dimorphic transition (Munro *et al*., 1998). There are so many anti-bacterial antibiotics which show target specificity to bacterial subcellular structures, while diversity of anti-fungal antibiotics is relatively narrow because fungi consist of eukaryotic cells like human body.

Therefore fungal cell wall can be the best target for antibiotics against fungi, because human does not have any fungal cell wall materials.

Coprinellus congregatus is a mushroom forming basidiomycete, and it is easy to grow in a complete agar medium. Induction of mushroom generation is also quite simple; incubation at 25°C in a regime of 15 h light/9 h dark cycle is enough to induce mushroom formation (Choi and Cho, 2005). This fungus generates mushrooms which become black ink droplets during their maturation, and this is why this mushroom is designated as an inky cap. When the autolyzed tissue and the liquid droplet were examined by the light and electron microscopy, the cell walls of basidia disintegrated during autolysis and no cell wall was observed in the liquid droplet (Choi and Cho, 2005). Since we would like to find out whether a chitinase was involved in the autolysis or not, a chitinase cDNA was isolated from the cells

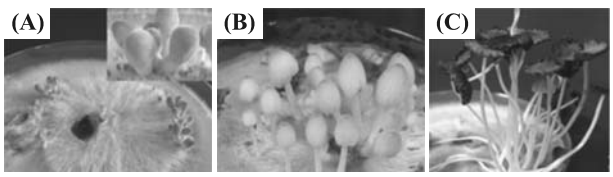

Fig. 1. Three different mushroom samples. (A) primordium (5 mm in length) at 2 h before the dark period on day 8. The inset showed higher magnification of the primordium; (B) young mushroom (1 cm in length) at the dark period initiation on day 8; (C) matured mushroom (in autolytic process) at 1 h after of the light period on day 9.

* To whom correspondence should be addressed.
(Tel) 82-33-250-8511; (Fax) 82-33-242-0459
(E-mail) htchoi@kangwon.ac.kr

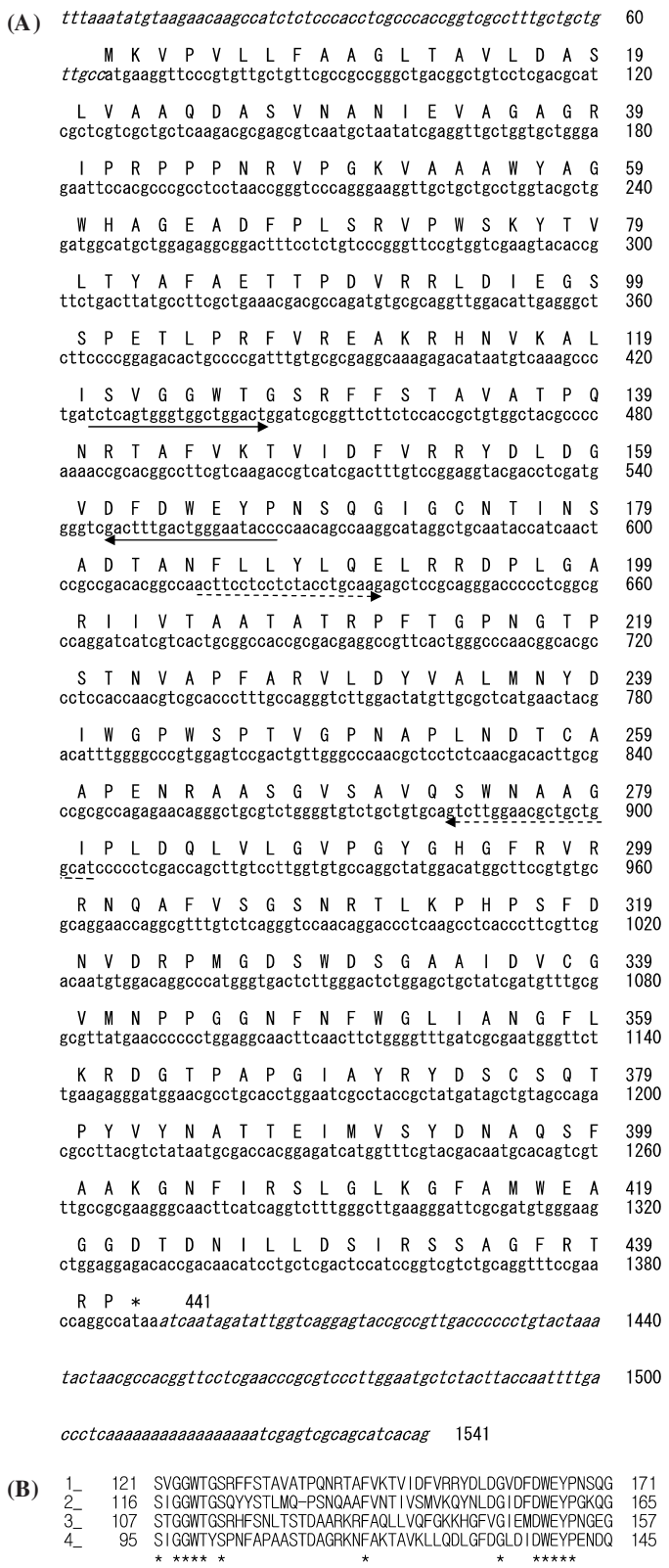

Fig. 2. (A) Cloned cDNA sequence and its deduced amino acid sequence of *chi1* of *Coprinellus congregatus*. Untranslated 5'- and 3'-regions are written in italics, and the * represents the stop codon. Two solid lines are the two primers used in the first PCR to get cDNA fragment, and two dotted lines represent the primers used in real time PCR. (B) Comparison of putative catalytic domain of Chi1 with three fungal chitinases. 1, *Coprinellus congregatus* chitinase (AM989926); 2, *Amanita muscaria* chitinase (AJ276119); 3, *Puccinia triticina* chitinase (AY267184); 4, *Aspergillus fumigatus* chitinase (1WNO_A). Stars represent the same amino acids in four different chitinases.

of autolyzed mushroom tissues, and its expression was also determined at the three different developmental stages. Here we report a chitinase that was expressed at the autolytic process in *C. congregatus*.

Growth conditions for mushroom development

C. congregatus Fries dikaryon (cc44×cc16) was grown on Emerson's YpSs plate (Difco) at 25°C for 4 days at dark place, and then transferred to an incubator with illumination of light (15 h) and dark (9 h) cycles. Three different fungal tissues from developmentally different stages of *C. congregatus* dikaryon were used as follows: primordium (ca. >5 mm in length) generated at 2 h before dark period on 8th day, young mushroom (1 cm in length) with closed pileus at time 0 of the dark period on 8th day, and matured mushroom at 1 h after transition to light period on 9th day [Fig. 1: part of Fig. 1 in Choi and Cho (2005) under the authors' permission]. Matured mushroom showing autolysis was used as the RNA source for chitianse cDNA cloning.

Cloning of chitinase cDNA

Fungal chitinases, which were the member of family 18 glycoside hydrolases, had conserved amino acid regions, and their nucleotide sequences were used in the PCR amplification of gene fragment (Choquer *et al*., 2007). Two domains which represented the box I and other region were used for synthesis of the degenerated primers: forward primer; 5'-TN TCARTNGGTGGHTGGACH-3', and reverse primer; 5'-GG RTAYTCCCARTCRAARTC-3'. Total RNA was isolated from matured mushroom tissue using RNeasy Plant Mini kit (QIAGEN, USA). The first strand of cDNA was synthesized from 1 μg of RNA using PowerScript Reverse Transcriptase (Promega, USA) following CapFishing cDNA isolation kit (Seegene, Korea), and PCR was performed using Taq polymerase with the two primers. In order to get full cDNA gene, 5'-region of the cDNA was synthesized using 5'-RACE-PCR primer and a specific reverse primer; 5'-GGGGTATT CCCAGTCAAAGTC-3', and 3'-region was amplified using 3'-RACE-PCR primer and a specific forward primer; 5'-TC AGTGGGTGGCTGGACTGGA-3'. Full length cDNA was generated by two-step PCR by following the manufacturer's protocol. We have successfully obtained the full cDNA (*chi1*) from *C. congregatus*, and this was reported to the EMBL nucleotide sequence database with the accession number AM989926. It consisted of 1,541 nucleotides and encoded 441 amino acids (Fig. 2A). The amino acid sequence of putative catalytic domain of Chi1 (441 amino acids) had well conserved amino acid sequences as in several fungal chitinase proteins. Chi1 showed high homology in putative catalytic domain (Aunpad and Panbangred, 2003) with different chitinase proteins of filamentous fungi, where stars represented the identical amino acids in four fungal chitinases (Fig. 2B). Chi1 of *C. congregatus* showed 56.9% identity with that of *Amanita muscaria* (AJ 276119), 43.1% with *Puccinia triticina* chitinase (AY267184), and 41.2% with that of *Aspergillus fumigatus* (1WNO_A). This domain of Chi1 showed 41.2% with *Coprinopsis cinereus* hypothetical protein (glycoside hydrolase family 18: locus CC1G_04870; from amino acid 198 to 246; data not shown), which has not yet been subjected to final NCBI review.

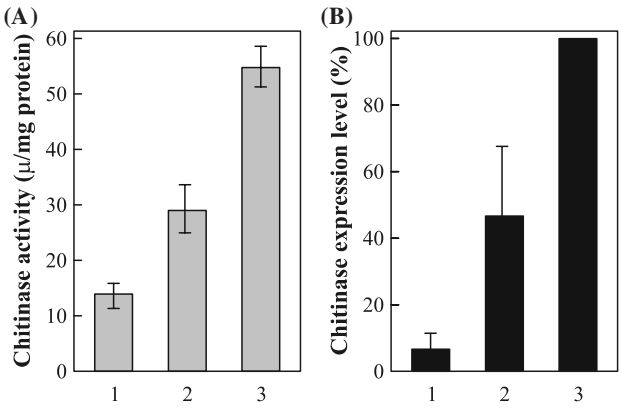

Fig. 3. Determination of chitinase activity and its expression in three developmentally different tissues of mushrooms. (A) Comparison of chitinase activity from three different fungal tissues. 1, primordium; 2, young mushroom; 3, matured mushroom. (B) Determination of chitinase expression by real-time PCR. Legends were same as in Fig. 3B.

Determination of chitinase activity and expression during the *C. congregatus* development

We have collected various mushroom tissues from three different developmental stages, as mentioned in growth condition section. Chitinase assay was performed using colloidal chitin as the substrate, and the generation of reducing sugar was compared for the relative chitinase activity. Bradford method was used for the protein concentration analysis. One unit of enzyme was defined as the amount of enzyme protein required generating 1 mg reducing sugar from the substrate. Chitinase activity from the matured mushroom tissue was higher than other two fungal tissues (Fig. 3A).

Total RNAs were isolated from samples using RNeasy Plant Mini kit (QIAGEN), and were used as the templates for the determination of chitinase gene expression by real-time PCR. Complimentary DNAs were synthesized from 3 μg of total RNAs from various tissues in 20 μl working volume. Real-time PCR was performed with 5 μl of cDNA mix, 12.5 μl of iTaq SYBR GreenSupermix (Bio- Rad), 1 μl of each chitinase-specific forward primer; 5'-ACTTCCTCC TCTACCTGCAAG-3' and reverse primer; 5'-ATGCCAGC AGCGTTCCAAGAC-3' in 25 μl reaction mixture. CT value of each sample was obtained by using ABI PRIAM 7000 SDS software, and the relative expression level of the laccase and MnP gene from each sample was compared with the expressed level of actin gene. The *chi1* was expressed most at the matured mushroom stage (Fig. 3B) when the mushroom tissues entered autolytic process. Its expression was 20 times or twice higher in matured mushroom with autolysis than in primordium or young mushroom, respectively. It is reasonable that chitinase is involved in the degradation of mushroom cell walls during the autolysis to generate the black liquid droplets. A chitinase (*chiB*) has been reported to be involved in the autolytic process of mycelia in *Aspergillus nidulans* under carbon source depletion (Yamazaki *et al*., 2007). Tissue cells of matured mushroom of *C. congregatus* also showed fast autolytic process for the spore dissemination purpose.

Many chitianses from different organisms have been reported to be involved in generation of anti-fungal materials against pathogenic fungi. Overexpression of an endochitinase gene from *Trichoderma reesei* in *T. atroviride* resulted in increased production of endochitinase, and showed biocontrol effect against a fungal pathogen, *Penicillium digitatum* (Deng *et al*., 2007). A transgenic lemon plant, expressing *T. harzianum* chitinase gene showed enhanced resistance to plant pathogenic *Phoma tracheiphila* and *Botrytis cinerea* (Gentile *et al*., 2007). Expression of a rice chitinase in taro (*Colocasia esculenta* (*L*.) Schott) resulted in improved tolerance to a fungal pathogen, *Sclerotium rolfsii* (He *et al*., 2008). Because *C. congregatus* is a saprophytic fungus, it does not synthesize chitinase enzyme to compete with other fungi. Even though chitinase participates in the hydrolysis of chitin polymer during the growth phase, the enzyme is highly required for the degradation of cell wall during the autolysis phase. This enzyme has potentials for anti-fungal activity when expressed in plants or dimorphic fungi.

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