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Biochemical Characterization of Chitinase 2 Expressed during the Autolytic Phase of the Inky Cap, *Coprinellus congregatus*

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Fungal cell walls consist of various glucans and chitin. The inky cap, *Coprinellus congregatus*, produces mushrooms at 25°C in a regime of 15 h light/9 h dark, and then the mushroom is autolyzed rapidly to generate black liquid droplets in which no cell walls are detected by microscopy. Chitinase cDNA from the mature mushroom tissues of *C. congregatus*, which consisted of 1,622 nucleotides (*chi2*), was successfully cloned using the rapid amplification of cDNA ends polymerase chain reaction technique. The deduced 498 amino acid sequence of Chi2 had a conserved catalytic domain as in other fungal chitinase family 18 enzymes. The Chi2 enzyme was purified from the *Pichia pastoris* expression system, and its estimated molecular weight was 68 kDa. The optimum pH and temperature of Chi2 was pH 4.0 and 35°C, respectively when 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside was used as the substrate. The K_m value and V_{max} for the substrate A, 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside, was 0.175 mM and 0.16 OD $min^{-1}unit^{-1}$, respectively.

***Keywords*:** chitinase, *C. congregatus*, mushroom autolysis

Introduction

Many fungi have a tissue-autolysis phase to complete their life cycle. The most prominent autolysis is pileus lysis shown in inky caps. Most filamentous fungi require a wall-lysis reaction during the growth phase to enlarge the surface area of their cell walls. The cell walls of filamentous fungi consist 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 organisms such as insects and crustaceans. Chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glycosidic linkages of the *N*-acetylglucosamine polymer chitin. There are two types of chitinases, such as endochitinase and exochitinase. Chitinases can also be grouped into two families, such as family 18 and family 19 glycoside hydrolases by their amino acid sequences (Henrissat, 1999). Chitinases in family 19 include most plant chitinases and *Streptomyces* chitinase, whereas fungal chitinases belong to family 18 (Henrissat, 1999). When opportunistically pathogenic fungi infect the human body, their cell morphologies undergo dimorphic changes to protect themselves from the host defense mechanism and to disseminate their progeny inside the host (Sundstrom, 2003). *Candida albicans* shows different chitin content during its dimorphic transition (Munro *et al*., 1998). Many antibacterial agents target bacterial subcellular structures, whereas the diversity of antifungal agents is relatively narrow, because fungi consist of eukaryotic cells like the human body. Therefore, the fungal cell wall can be the best target for antifungal agents, because humans have no fungal cell wall materials.

Coprinellus congregatus is a mushroom-forming basidiomycete that is easy to grow in complete agar medium. Inducing the generation of mushrooms is also quite simple; incubation at 25°C in a regime of 15 h light/9 h dark is sufficient (Choi and Cho, 2005). This fungus generates mushrooms that become black ink droplets during maturation, which is why this mushroom is designated the inky cap. When the autolyzed tissue and the liquid droplets are examined under light and electron microscopy, the cell walls of basidia disintegrate during autolysis, and no cell wall is observed in the liquid droplets (Choi and Cho, 2005). As we were interested in determining which type of chitinase is expressed during autolysis, and since it was very difficult to get large amount of autolyzed mushroom tissues under the lab condition, cDNA of the chitinase which was expressed at this phase was necessary to obtain the enzyme for the next biochemical characterizations. The expression of isolated chitinase 2 (*chi2*) was determined at the three different developmental stages. The Chi2 expressed at the maturation phase was obtained using the *Pichia pastoris* expression system, and the biochemical characteristics of Chi2 were examined.

Materials and Methods

Growth conditions for mushroom development

C. congregatus Fries dikaryon (cc16 × cc44) was grown on Emerson's YpSs plates (Difco, USA) at 25°C for 4 days in the dark and then transferred to an incubator with illumination of 15 h light and 9 h dark. Three different fungal tissues from different developmental stages of the *C. congregatus* dikaryon were used: primordium (ca. >5 mm in length) generated at 2 h before the dark period on day 8, young mushrooms (1 cm in length) with a closed pileus at time 0 of the dark period on day 8, and mature mushrooms at 1 h after transition to the light period on day 9 (See Fig. 1 of

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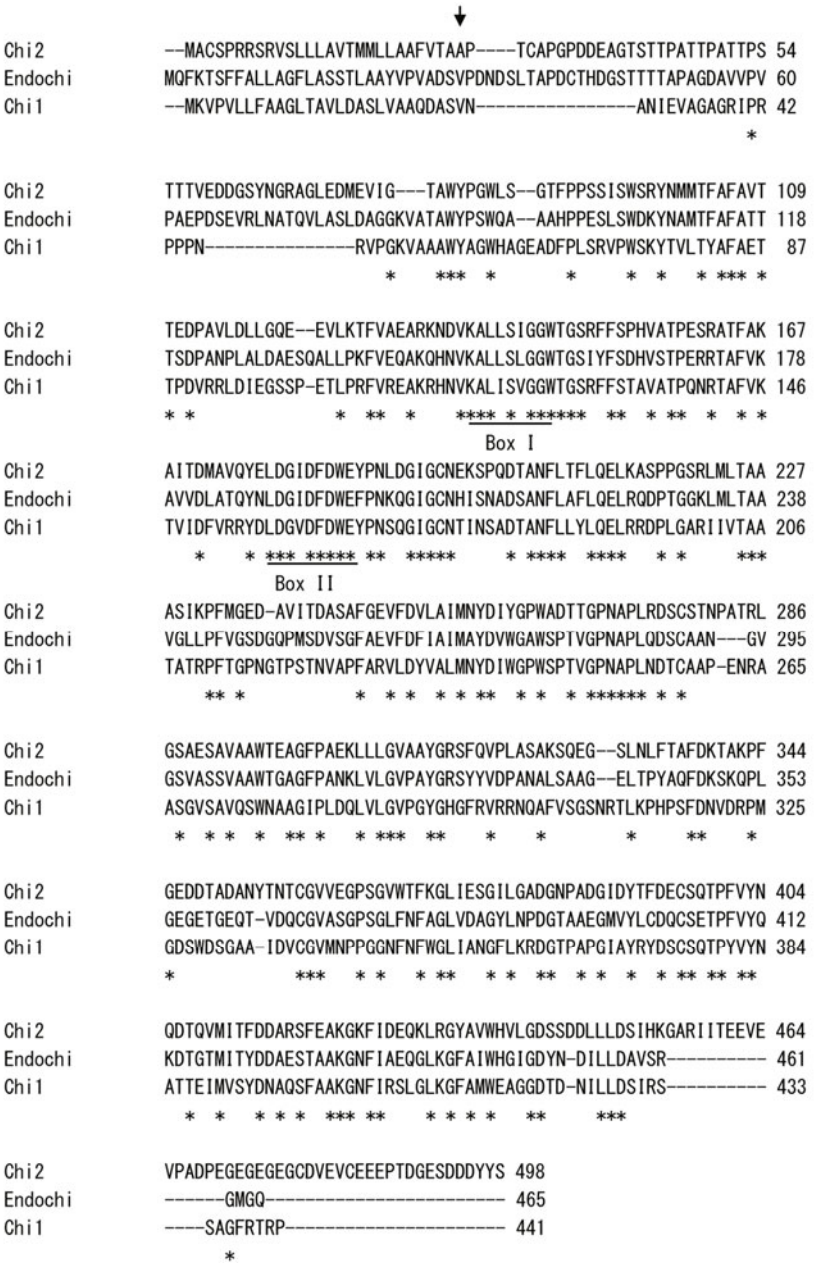

Fig. 1. Comparison of amino acid sequences of chitinase 2 (Chi2) with other chitinases of inky caps. Identical amino acids from all proteins are shown with stars on the bottom line. Boxes I and II, which represent chitin-binding and catalytic domains, respectively, are underlined. The cleavage site of the signal peptide is indicated by an arrow. Chi2, in this experiment (JQ413429); Chi1, *C. congregatus* chitinase 1 (AM989926); Endochi, endochitinase of *C. cinerea* (EAU81461.1).

Choi and Cho, 2005). Mature mushrooms undergoing autolysis were used as the RNA source for chitinase cDNA cloning.

Chitinase 2 cloning and confirmation of expression during autolysis

Fungal chitinases, which are members of family 18 glycoside hydrolases, have conserved amino acid regions, and their nucleotide sequences have been used in the polymerase chain reaction (PCR) amplification of gene fragments (Choquer *et al.*, 2007). Two domains representing box I and II were used for synthesis of the degenerated primers: forward primer 5′-TNTCARTNGGTGGHTGGACH-3′, and reverse primer 5′-GGRTAYTCCCARTCRAARTC-3′ (Lim and Choi, 2009). Total RNA was isolated from mature mushroom tissue using the RNeasy Plant Mini kit (Qiagen, USA). First-strand cDNA was synthesized from 1 μg of RNA using PowerScript Reverse Transcriptase (Promega, USA) using a CapFishing cDNA Isolation kit (Seegene, Korea), and PCR was performed using Taq polymerase with the two primers. To obtain the full cDNA gene, the 5′-region of the cDNA was synthesized using 5′-rapid amplification of cDNA ends (RACE)-PCR primers and a specific reverse primer (5′-TGGACAGCCATG TCGGTGAT-3′), and the 3′-region was amplified using a 3′-RACE-PCR primer and a specific forward primer (5′-TCA AGGTTCTTTTCACCCCATGT-3′). Full length cDNA was generated by two-step PCR following the manufacturer's protocol. Several specimens from different developmental stages such as hyphae, primordia, young mushrooms, and autolysing mushrooms were collected to isolate RNAs for

reverse transcription-PCR and real time RT-PCR to confirm that *chi2* was expressed most at the mushroom autolysis phase. Two specific primers were used in these experiments: forward primer (qF) 5′-TCAAGGCCTCTCCCCCTGGTT-3′ and reverse primer (qR) 5′-CGAGTCTCGTTGCAGGGTT GG-3′. Real time PCR was performed with 5 µl of cDNA mix, 12.5 µl of iTaq SYBR GreenSupermix (Bio-Rad, USA), 1 µl of each *chi2*-specific primers in a 25 µl reaction mixture. Actin gene expression was also determined from the four different specimens as a control.

Construction of the *chi2* expression vector with *P. pastoris* vector and purification of Chi2 using a histidine tag

Vector, pPICZB, was used to construct the *chi2* expression vector. In order to construct the *chi2*-expression vector, the *Eco*RI and *Sal*I linkers were attached at the forward and reverse primer respectively, which included the full ORF and histidine tag. The expression vector (pPICZBchi2) was introduced into the *P. pastoris* GS115 strain following the manufacturer's protocols using zeocin resistance as the selectable marker. The genetic transformants were grown in complete medium, and the cells were transferred to BMGY (yeast extract 0.5%, peptone 1%, 100 mM sodium phosphate buffer; pH 6.0, yeast nitrogen base 1.34%, biotin 0.00004%, glycerol 1%). Whole cells were transferred to BMMY (same as BMGY but methanol 0.5% instead of glycerol 1%), and methanol (final concentration, 0.5%) was added every 24 h to induce *chi2*. The culture supernatant of the *chi2*-induced transformant was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with that of the un-induced culture.

Expressed Chi2 was purified by the histidine tag using His·Bind Agarose Resin (Elpis Biotech, Korea) following the manufacturer's protocol. The molecular weight of the purified protein was analyzed by 10% SDS-PAGE with protein molecular weight markers. The N-terminal amino acid sequence of the purified chitinase was analyzed by gel permeation chromatography (Procise 491 HT protein sequencer, Applied Biosystems, USA) at the protein analysis lab, Seoul Center of Korea Basic Science Institute. The chitinase assay was performed using Chitinase Assay Kit (Sigma, USA), which consisted of three different substrates, such as 4-nitrophenyl N,N'-diacetyl-β-D-chitobioside (substrate A), 4-nitrophenyl N-acetyl-β-D-glucosaminide (substrate B), and 4-nitrophenyl β-D-N,N',N''-triacetylchitotriose (substrate C). Enzyme activity was determined with 50 µl of each substrate containing 5 µl of enzyme solution with 45 µl of buffer (50 mM acetate buffer, pH 5.0) at 35°C for 10 min. The reaction was stopped by adding 100 µl of stop solution, and the absorbance was read at 405 nm. Protein concentrations were determined by following the Lowry method. Enzyme activity was expressed as the absorbance increase of 0.1 at 405 nm/min/mg protein under the above reaction conditions.

Biochemical characterization of the purified Chi2

Substrate A (MW, 545.5 g) was dissolved in the assay buffer provided by Sigma at a concentration of 0.2 mg/ml. The optimum pH was determined in the range from 3.0 to 8.0 with citrate-phosphate buffer (pH 3.0–7.0) and phosphate buffer (pH 7.0–8.0) at 35°C for 10 min, and the optimum temperature was determined from 5°C to 50°C at pH 4.0 for 10 min. The K_m and V_{max} for substrate A were determined by measuring initial velocity at 35°C, pH 4.0, with five different substrate concentrations (0.2–1.0 mM). All kinetic experiments were performed three times, and the data were fitted to hyperbolae using the Michaelis-Menten equation.

Results and Discussion

Full-length *chi2* cDNA and confirmation of expression during autolysis

A chitinase cDNA was synthesized by RACE-PCR using RNA isolated from the autolysing mushroom tissue as the cDNA source. It was designated as *chi2* and was reported to the EMBL nucleotide sequence database under accession number JQ413429. *Chi2* consisted of 1,622 nucleotides and encoded 498 amino acids. Chi2 had conserved domains that were putative chitin-binding and catalytic domains (Aunpad and Panbangred, 2003) (Fig. 1). When the Chi2 amino acid sequence was compared with that of other chitinases, it showed 44% identity with Chi1 (AM989926) of *C. congregatus* and 45% of identity with endochitinase (EAU81461.1) of *Coprinopsis cinerea*.

RT-PCR and real-time PCR revealed that *chi2* was expressed most at the mature mushroom stage (Figs. 2A and 2B) when the mushroom tissues entered the autolytic process. Its expression was 20 times or twice higher in mature mushrooms showing autolysis than that in primordia or young mushrooms, respectively. It is reasonable to assume that chitinase is involved in the degradation of mushroom cell walls during 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 mature *C. congregatus* mushroom also showed a fast autolytic process for spore dissemination.

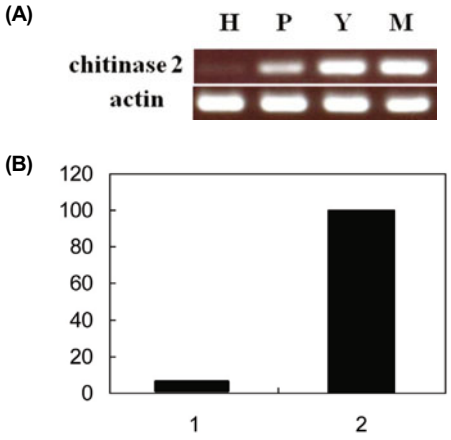

Fig. 2. Determination of chitinase 2 (*chi2*) expressions in three developmentally different mushroom tissues and hyphal tips by RT- PCR (A) and real-time PCR (B). H, hypha; P, primordium; Y, young mushroom; M, mature mushroom.

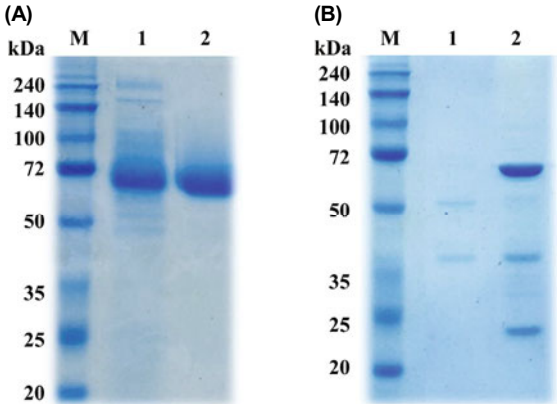

Fig. 3. PAGE analysis for chitinase 2 (Chi2) synthesis using the *P. pastoris* expression system (A), and purification of Chi2 by histidine tag (B). (A) M, molecular weight marker; 1, culture supernatant of recipient strain of *P. pastoris*; 2, culture supernatant of pPICZBchi2 transformant. (B) M, molecular weight marker; 1, dialyzed concentrate of culture supernatant from pPICZBchi2 transformant; 2, purified Chi2 through histidine-tag resin.

Purification of Chi2 generated from the *P. pastoris* expression system

The genetic transformants with pPICZBchi2 were grown in BMMY medium, and each strain was induced with methanol to obtain Chi2. Chitinase activity was detected only from the stain with the pPICZBChi2 vector on day 2 and was confirmed by a thick band that appeared in the culture supernatant on SDS-PAGE (Fig. 3A). The N-terminal amino acid sequence of purified Chi2 was A-P-T-C-A, which was identical with amino acids 28–32. This result indicated that the signal peptide of Chi2 was active in *P. pastoris*, and that active Chi2 was successfully secreted from the yeast cells. Chi2 was purified using the histidine tag and resulted in a single band on SDS-PAGE (Fig. 3B). It was very difficult to purify enzymes from the autolysing tissues, since we could only generate small amount of autolysing mushroom tissues under lab conditions. We could obtain plenty amount of enzyme proteins for their biochemical characterization by this heterologous expression technique.

Biochemical characteristics of Chi2

Substrate A was used to determine the biochemical characteristics. The optimum pH was 4.0, and the optimum temperature was 35°C (Figs. 4A and 4b). The K_m value for substrate A was 0.175 mM, and the V_{max} was 0.16 (OD $min^{-1}unit^{-1}$) (Fig. 4C). These parameters of *C. congregatus* Chi2 were not compared with other fungal chitinases, because substrate A was not the actual chitinase substrate. Chi2 showed a color reaction generated by the liberation of 4-nitrophenol only with substrate A, indicating that Chi2 was an exochitinase. Many different enzymes are present as fungal cell walls are composed of diverse polysaccharides and proteins. Therefore, Chi2 is not the only enzyme involved with mushroom autolysis in *C. congregatus*. Another chitinase (*chi1*) has been reported in this fungus, and there was a good possibility that Chi1 and Chi2 work together with other glycosyl hydrolases. Even though a chitinase participates in the hydrolysis of a chitin polymer during the growth phase, chitinases are required for cell wall degradation during the autolytic process in *C. congregatus*.

Many chitinases from different organisms are involved in the generation of antifungal material against pathogenic fungi. Overexpression of an endochitinase gene from *Trichoderma reesei* in *T. atroviride* results in increased production of endochitinase, and shows biocontrol against the fungal pathogen, *Penicillium digitatum* (Deng *et al.*, 2007). A transgenic lemon plant expressing the *T. harzianum* chitinase gene shows 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) results in improved tolerance to a fungal pathogen, *Sclerotium rolfsii* (He *et al.*, 2008). Expression of *chiB* during development of

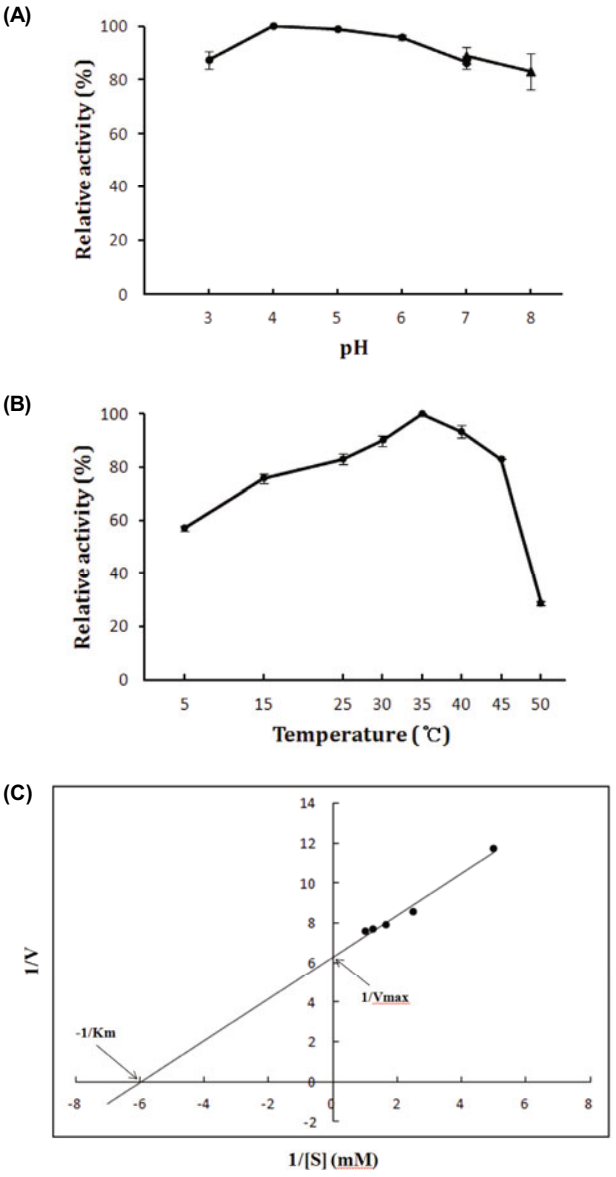

Fig. 4. Biochemical characteristics of the purified chitinase 2 (Chi2). (A) optimum pH. Closed circle, citrate-phosphate buffer; closed triangle, phosphate buffer; (B) optimum temperature; (C) K_m and V_{max}.

A. nidulans is regulated by the FluG-BrlA signal, which is the key regulator for asexual reproduction (Pócsi *et al*., 2009). Instead of *chi2* expression in other fungal species to examine their growth regulation, Chi2 itself will be used in the growth inhibition experiments of several pathogenic fungi by adding to the fungal growth media.

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