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NOTE

Antifungal Chitinase against Human Pathogenic Yeasts from *Coprinellus congregatus*

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The inky cap, *Coprinellus congregatus*, produces mushrooms which become autolyzed rapidly to generate black liquid droplets, in which no cell wall is detected by microscopy. A chitinase (Chi2) which is synthesized during the autolytic phase of *C. congregatus* inhibits the growths of *Candida albicans* and *Cryptococcus neoformans* up to 10% at the concentration of 10 μg/ml, about 50% at concentration of 20 μg/ml, and up to 95% at the concentration of 70 μg/ml. Upon treatment these yeast cells are observed to be severely deformed, with the formation of large holes in the cell wall. The two yeast species show no growth inhibition at the concentration of 5 μg/ml, which means the minimum inhibitory concentrations for both yeast species are 10 μg/ml under these experimental conditions.

***Keywords*:** antifungal chitinase, *Coprinellus congregatus*, human pathogenic yeasts

The cell wall of filamentous fungi consists of diverse polymers such as α- and β-glucans, other sugar polymers and chitins. Chitin, the 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. A chitinase (ChiA) has been reported that it is localized at polarized growth sites in *Aspergillus nidulans* (Yamazaki *et al*., 2008). Another chitinase (*chiB*) has been reported to be involved in the autolytic process of mycelia in *A. nidulans* under carbon source depletion (Yamazaki *et al*., 2007). Therefore, fungal chitinases show dual functions in growing phase as well as in autolysing phase. Chitinases have also been reported that they can inhibit many fungal growths (Brzezinska and Jankiewicz, 2012; Hammami *et al*., 2013).

Coprinellus congregatus is a mushroom forming basidiomycete, and 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 induces mushroom formation (Choi and Cho, 2005). This fungus generates mushrooms which become black ink droplets during their maturation, and that is why this fungus has the common name of 'inky cap'. When the autolyzed tissue and the liquid droplet were examined under both light and electron microscopy, the cell walls of basidia could be observed to disintegrate during autolysis, and no cell wall was observed in the liquid droplet (Choi and Cho, 2005). We have successfully isolated a chitinase cDNA from the autolyzing mushroom tissue, and constructed an expression vector of the chitinase gene (*chi2*) (Kang *et al*., 2013). Biochemical characteristics of the heterologously expressed Chi2 from *Pichia pastoris* were reported (Kang *et al*., 2013). When Chi2 was applied to liquid culture of *Saccharomyces cerevisiae*, yeast growth was inhibited and a large hole appeared in Chi2-treated cells (unpublished data). Here, we would like to report the effect of Chi2 on the growth inhibition of human pathogenic yeasts.

Purification of Chi2 was followed as described previously (Kang *et al*., 2013), and the brief procedure was as follows: cDNA of Chi2 was constructed as an expression vector using Pichia expression vector (pPICZB), and it was introduced into *P. pastoris* to obtain the Chi2 producing strain. The genetic transformant was induced to generate Chi2, and the Chi2 in the culture supernatant was purified by the histidine tag using His·Bind Agarose Resin (Elpis Biotech, Korea) following the manufacturer's protocol. There was only 1 protein band on the SDS-gel when the purified Chi2 was examined by the PAGE analysis as reported previously (Fig. 3 of Kang *et al*., 2013) (Fig. 1).

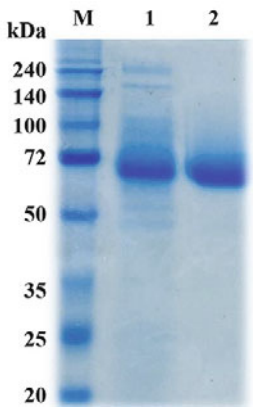

Fig. 1. PAGE analysis of purified Chi2. M, molecular weight marker; 1, dialyzed concentrate of culture supernatant from pPICZBchi2 transformant; 2, purified Chi2 through histidine-tag resin.

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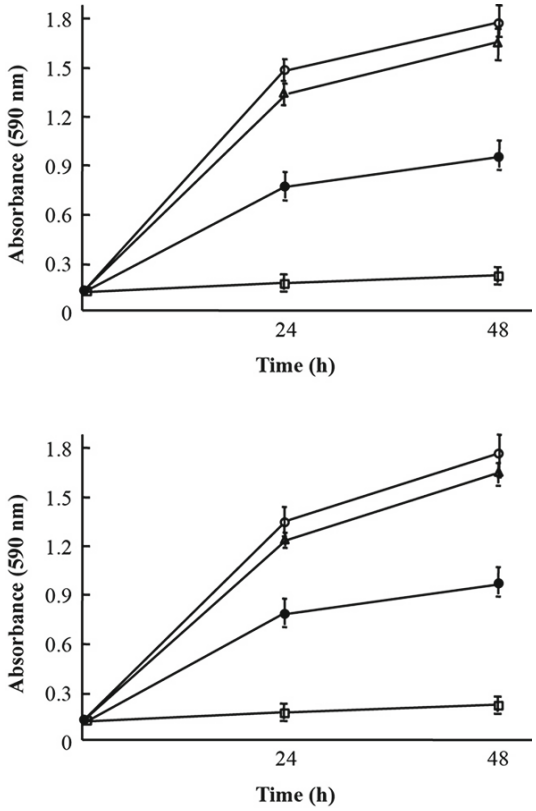

Fig. 2. Growth inhibition of the two pathogenic yeast species by addition of Chi2. Top, *C. albicans*; Bottom, *C. neoformans*. Open circle, control; Triangle, Chi2 10 μg/ml; Closed circle; Chi2 20 μg/ml; Square, Chi2 70 μg/ml.

In order to examine the antifungal activity of Chi2 against two human pathogenic yeasts, *Candida albicans* and *Cryptococcus neoformans* were selected. *Candida albicans* KCTC 1940 was grown on YMPG plates (yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g, agar 16 g, d-H_2O 1 L) at 25°C, and was then transferred to a broth with the same composition. Chi2 was added to the broth culture (2 ml in 15 ml conical tube) at concentrations of 5 μg/ml, 10 μg/ml, 20 μg/ml or 70 μg/ml. *Cryptococcus neoformans* (serotype A, H99) was grown on YMPG plates at 30°C, and was then transferred to the same broth medium. Chi2 was added at the same concentrations as were added to *C. albicans*. The growth of each pathogenic yeast species was analyzed by measuring the absorbance at 590 nm. Growths of *C. albicans* and *C. neoformans* in liquid media were inhibited by 10% by 10 μg/ml of Chi2, 40–50% by 20 μg/ml of Chi2 and were inhibited by more than 95% by 70 μg/ml of Chi2, as dose-dependent response (Fig. 2). However 5 μg/ml of Chi2 showed no growth inhibition for both of the yeast species (data not shown), and the minimum inhibitory concentrations of Chi2 against two pathogenic yeast species were 10 μg/ml under these experiment conditions.

Yeast cells were collected by centrifugation (6,000×g) after 2 days of incubation, and were fixed with 4% glutaraldehyde solution at 4°C for 2 h. Fixed cells were dehydrated through a series of ethanol, and were then substituted with isoamyl acetate. After drying the specimens, they were sputter-coated with Au-Pd. Chi2-treated cells were analyzed using Variable Pressure Field Emission Scanning Electron Microscope (VP-FE-SEM; Carl-Zeiss, Germany) in Chunchon Center of Korea Basic Science Institute. Both yeast cells were severely damaged; a large hole was observed in each cell of Chi2-treated *C. albicans*, and some Chi2-treated *C. neoformans* populations were observed to have been bisected through the effects of the treatment (Fig. 3). Yeast cells were observed to have bud scars resulting from the cell division, and chitins were localized more at the bud scar regions. The induction of a chitinase (*chi1*) of *C. congregatus* in *S. cerevisiae* showed decreased growth (Lim and Choi, 2010), which meant that chitinase could inhibit fungal growth. Chitins are also important components in the cell walls of dividing young cells of *C. albicans* and *C. neoformans*, and therefore, it was reasonable that chitinase inhibited the growths of both fungi and destroyed the growing yeast cells by inhibition of cell wall generation. Recently Tsirilakis *et al.* (2012) reported an interesting result that an inhibitor (methylxanthine) of family 18 chitinase which included fungal chitinases (Rao *et al.*, 2005) inhibited the growth of *C. neoformans*, but not that of *C. albicans*. The authors suggested that the differences in growth inhibition of two yeast species might reflect structural differences among the fungal chitinases, and that this resulted in the lack of growth inhibition. However, Chi2 showed very good inhibition against both pathogenic yeast species.

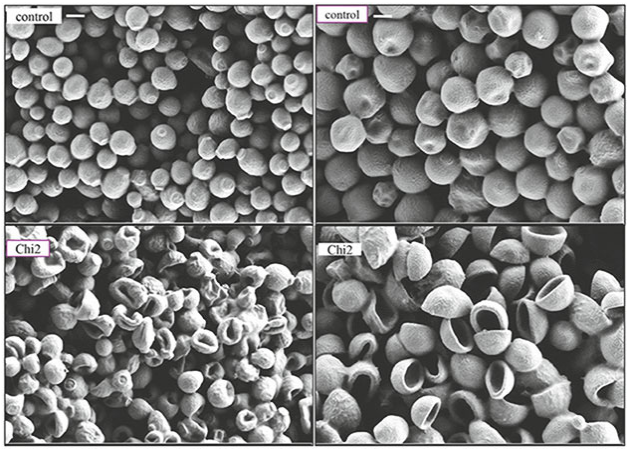

Fig. 3. Scanning electron microscopy shots of Chi2-treated yeast cells. Left, *C. albicans*; Right, *C. neoformans*. Control, no Chi2 addition; Chi2, 70 μg/ml. White scale bar, 3 μm.

The dimorphic change of *C. albicans* is the most important physiological character of pathogenicity of this fungus. *C. albicans* should form a biofilm to set up localization at the yeast infected area, and filament formation is a prerequisite for biofilm generation (Ramage *et al.*, 2002). Since *C. albicans* biofilm is associated with drug resistance (Finkel and Mitchell, 2011), and hyphal formation has been reported to increase chitin content in the hyphal cells in *C. albicans* (Munro *et al.*, 1998), Chi2 could provide a solution to the inhibition of dimorphic changes in this fungus, by removal of chitin from the cell wall. In this study, Chi2 gave positive

results as an antifungal material against pathogenic yeasts. Chi2 can be used as a natural preservative in cosmetics and ointments.

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