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Antifungal Activity of Chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9

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Abstract Two chitinolytic fungal strains, *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9, were isolated from soil samples of Korea and Vietnam, respectively. DY-59 and VS-9 crude chitinases secreted by these fungi in the 0.5% swollen chitin culture medium had an optimal pH of 4 and the optimal temperatures of 40°C and 60°C, respectively. Enzymatic hydrolysis products from crab swollen chitin were *N*-acetyl-β-D-glucosamine (GlcNAc) by DY-59 chitinase, and GlcNAc and *N*, *N*′-diacetylchitobiose $(GlcNAc)_2$ by VS-9 chitinases. The chitinases degraded the cell wall of *Fusarium solani* hyphae to produce oligosaccharides, among which GlcNAc, $(GlcNAc)_2$, and pentamer $(GlcNAc)_5$ were identified by high-pressure liquid chromatography. DY-59 and VS-9 chitinases inhibited *F. solani* microconidial germination by more than 70% and 60% at final protein concentrations of 5 and 27 μg mL^{-1}, respectively, at 30°C for 20 h treatment.

Introduction

Chitinases are produced by a wide range of organisms including viruses, actinomycetes, bacteria, fungi, nematodes, insects, spiders, plants, and vertebrates [7, 8, 11, 13, 17]. Among the chitinase-producing organisms, fungi are believed to produce various isoforms of chitinases with different biophysical functions. In fungi, chitinases play the important biological and physiological roles: lysis of the cell walls (separation of cells after division, hypha autolysis), nutritional requirements, morphogenetic formation (sporulation, spore germination, hyphal growth), and antagonistic actions against other microorganisms [18]. These enzymes can be classified as endochitinases and exochitinases. Exochitinases can be subdivided to chitobiosidase and *N*-acetyl-β-D-glucosaminidase. Most of the fungal chitinases are multidomain structures with a wide molecular mass range from 27 to 190 kDa [5, 6, 11].

Among their applications, chitinolytic enzymes have been studied as potential antifungal agents against chitin-bearing plant pathogens because the enzymes play a key role in the mechanism of parasitic entry into host cells [5, 12]. Because the fungal cell wall has a cross-linked complex structure composed of chitin, glucans, and other polymers [2], enzymes that hydrolyze these components play a significant role in cell wall lysis of the pathogens [1, 4]. The objectives of this research were focused on (1) isolation of chitinase-producing fungi and (2) suppression in microconidial germination of *Fusarium solani* by these fungal chitinases. *F. solani* is a phytopathogen causing diseases in many agricultural crops throughout the world [2].

Materials and Methods

DY-59, VS-9 and *Fusarium solani* Strains

Antagonistic fungi were isolated from the soil samples obtained from Korea and Vietnam on peptone–rose bengal agar medium containing 5 g peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 10 g dextrose, 30 mg rose bengal, 20 g agar, and 50 mg streptomycin in 1 L water [16]. *F. solani* was

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isolated from cucumber *Meloidogyne* root-knot samples [3, 15].

Preparation of DY-59 and VS-9 Crude Chitinases

DY-59 and VS-9 isolates were cultured in 0.5% swollen chitin broth medium at 25°C and 150 rpm for 7 days. The culture medium was centrifuged at 6000 rpm for 30 min, and then filtered through No. 2 Whatman filter paper followed by filtration through a 0.2-μm membrane (Nalgene). The filtrate obtained was analyzed for chitinase activity, protein content, and antifungal activity [19].

Enzyme Assay

The chitinase and *N*-acetyl-*β*-D-glucosaminidase activity was estimated by reducing sugar groups with swollen chitin as the substrate [9] and *p*-nitrophenol released from *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide, respectively [10].

Anti-germination Activity

F. solani spore suspension was harvested from PDA plates after being cultured at 25°C for 7 days by adding a solution containing 0.05% glucose and 0.05% KH_2PO_4 [20]. To estimate the anti-germination activity of DY-59 and VS-9 chitinases on *F. solani* conidia, volumes of 0, 20, 60, and 100 μL of enzyme preparation were added to 100 μL of *F. solani* spore suspension containing 4.2×10^6 microconidia mL^{-1} and the final volume was adjusted to 200 μL using heated enzyme. The mixtures were incubated at 30°C and the number of the germinated microconidia was observed and counted through a microscope.

Fungal Cell Wall Hydrolysis Activity

F. solani biomass was prepared by vacuum filtration of fungal potato dextrose broth cultured for 7 days. *F. solani* cell walls were digested at 40°C for 24 h by the DY-59 and VS-9 enzyme preparation in a mixture containing 1% hypha biomass suspension and crude enzyme (2:1, v/v) with shaking at 70 rpm. Separation of *N*-acetyl-chitooligosaccharides [$(GlcNAc)_n$, n = 1 ~ 6] was performed on a column NH_2P-50 4E (Shodex, Japan) by using a mobile phase of distilled water: acetonitrile, 30:70 (v/v) at a flow rate of 1 mL min^{-1}. Elution of *N*-acetylchitooligosaccharides from column was monitored at 210 nm [10].

Results and Discussion

Isolation of DY-59 and VS-9 and the Characteristics of Their Chitinases

The DY-59 and VS-9 chitinolytic fungi were screened from more than 100 fungal strains isolated from soil samples obtained from Korea and Vietnam [15]. These fungal strains were identified and assigned as *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9 by morphological and phylogenetic analysis. *Rhizopus microsporus* VS-9 is a newly reported species that produces chitinases.

These fungal strains exhibited clear zones on swollen chitin plates, where the VS-9 strain simultaneously formed a clear zone with growth of colonies but the DY-59 strain produced a clear zone by the time the fungal colonies had

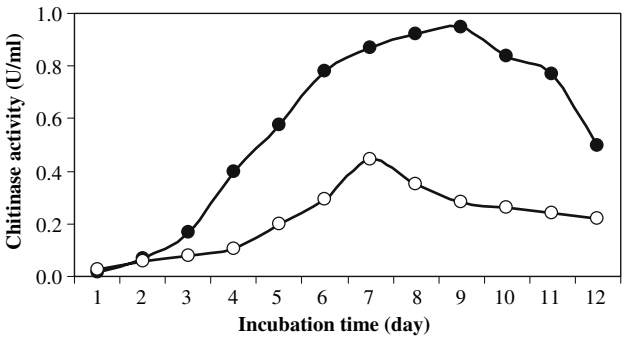

Fig. 1 Time courses of chitinase production of the DY-59 (•) and VS-9 (○) fungi. The fungi were grown in a broth medium containing 0.5% swollen chitin in a 250-mL Erlenmeyer flask at 25°C for 12 days

Table 1 Characteristics of chitinolytic fungi *T. aureoviride* DY-59 and *R. microsporus* VS-9 in 0.5% swollen chitin cultural medium

Isolates	Clear zone formation on chitin plate	Reducing sugar in broth culture (μmol/mL)	Chitinase activity (U/mL/h)	GlcNAcase (U/mL/min)
T. aureoviride DY-59	++	2.309	1.25	1.530
R. microsporus VS-9	+++	0.580	0.45	0.023

-: not clear; +: slightly clear; ++: clear; +++: very clear

GlcNAcase: *β*-(1,4)-*N*-acetyl-glucosaminidase (EC 3.2.1.30)

fully grown on the culture plates. These fungi produced reducing sugar and chitinases in swollen chitin broth medium (Table 1). The chitinase activities of DY-59 and VS-9 strains with 1% swollen chitin substrate were 1.25 and 0.45 U mL^{-1}, respectively. The *N*-acetyl-*β*-D-glucosaminidase activities of the DY-59 and VS-9 strains with 5 mM *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide (pNP-GlcNAc) substrate were 1.53 and 0.023 U mL^{-1}, respectively. The time course of chitinase activity of the DY-59 and VS-9 strains in 0.5% swollen chitin liquid medium for 12 days showed that the enzyme activity increased from low levels in early stages of cultivation to higher levels at later stages, and reached the highest activity for 7 days and 9 days, respectively (Fig. 1). The optimal temperature and pH of the DY-59 and VS-9 crude chitinases was at 40°C and 60°C and at pH 4, respectively. Six materials of chitin, chitosan, and their derivatives were used for determining substrate specificities of DY-59 and VS-9 chitinases, and

Table 2 Inhibition of *F. solani* microconidial germination by crude chitinases

DY-59			VS-9		
Amount of enzyme (μg mL^{-1})	Hours after incubation		Amount of enzyme (μg mL^{-1})	Hours after incubation	
	8 h	20 h		8 h	20 h
0	0^{d}	0^{c}	0	0^{c}	0^{c}
5	24.5^{c}	76.5^{b}	9	16.9^{b}	33.5^{b}
15	46.0^{b}	91.3^{a}	27	22.1^{b}	67.6^{a}
25	64.7^{a}	94.5^{a}	45	80.3^{a}	89.6^{a}

The inhibition activity was calculated using the flowing equation [14]: inhibition % = (C – E)/C × 100; where C is the number of germinated conidia in the control; and E is the number of germinated conidia in the enzyme treatment. Values in the vertical column followed by different superscripted letters are significantly different at $P \leq 0.05$ as determined using Tukey's Studentized Range (HSD) test

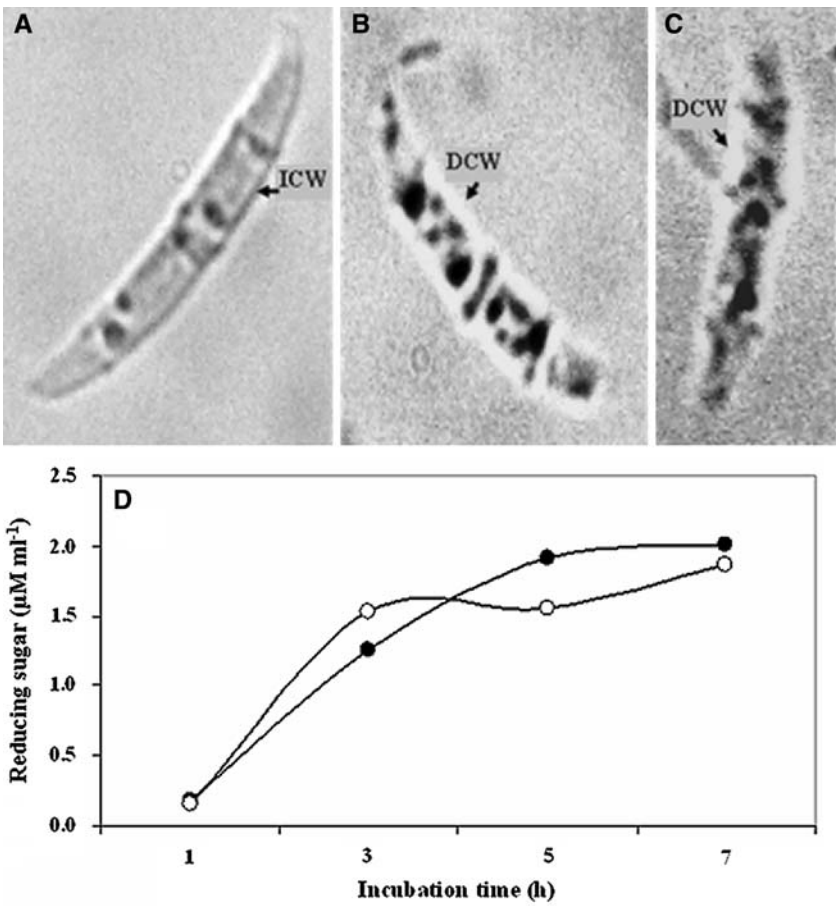

Fig. 2 Lysis of cell wall of *F. solani* macroconidia by the DY-59 and VS-9 chitinases. The mixture of enzyme and conidia suspension was incubated at 30°C for 20 h. Control **(A)**, DY-59 chitinase **(B)**, VS-9 chitinase **(C)**, and reducing sugar from *F. solani* hypha cell walls **(D)**. A reaction mixture contained 900 μL of 1% hypha biomass in sodium acetate buffer (pH 5) and 100 μL of crude enzyme from DY-59 (•), and VS-9 (○). ICW, intact cell wall; DCW, digested cell walls

soluble chitin (DA 50%) was the most suitable substrate for two chitinases. Hydrolysis products from crab swollen chitin were: GlcNAc by DY-59 chitinase, and GlcNAc and $(GlcNAc)_2$ by VS-9 chitinases (data not shown). Crude enzymes of the DY-59 and VS-9 strains were separated to 5 and 7 protein bands, respectively, by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Chitinase activity staining with 0.01% glycol chitin as a substrate in the gel revealed approximately 52-kDa endochitinase from DY-59 and 64 and 59 kDa endochitinases from VS-9 (data not shown). Most chitinolytic fungi produce more than one kind of isoform, for example, four *Trichoderma harzianum* endochitinases of 52, 42, 33, and 31 kDa [11]. Fungal chitinases belong to family 18 with a wide range of molecular weight from 27 to 190 kDa.

Anti-germination and Hyphal Cell Wall Hydrolysis Activities of the Crude Chitinases

Genus *Trichoderma* is well-known as biological control agent for several plant diseases [4, 7]. The DY-59 showed higher enzyme activity compared with *Rhizopus* genus VS-9 (Table 1). An in vitro experiment to investigate competition between *T. aureoviride* DY-59 and *R. microsporus* VS-9 with *F. soloni* showed that DY-59 inhibited the growth of *F. soloni* colonies and formed a space between two fungi, whereas VS-9 inhibited less, and the hyphae of two fungi grew across each other (data not shown).

The crude enzyme of the DY-59 and the VS-9 strains exhibited inhibitory effects on spore germination (Table 2) and degradation of cell walls (Fig. 2). The chitinases of the DY-59 and VS-9 had the IC_{50} values estimated at 16.5 and 36.9 μg mL^{-1} of enzyme concentration at 8 h treatment, respectively (Table 2). The DY-59 and VS-9 chitinases at 8 h treatment showed more than 50% germination inhibition of *F. solani* spore germination at protein concentrations of 25 and 45 μg mL^{-1}, respectively, and at 20 h treatment more than 70% and 60% germination inhibition at protein concentrations of 5 and 27μg mL^{-1}, respectively. In fungi, the growing hypha tip may be especially sensitive to chitinases because fungal cell walls contain chitin and glucans as the skeletal component [2]. Hyphae and spores grow by elongating at the tips and in which intracellular chitinases possibly function in loosening the cell wall to enable pressure for extending the hypha tip at the apex [11]. Therefore, the chitinases supplied externally may be considered as the factor that solubilizes further the hypha tip and results in inhibition of spore germination. The inhibitory effect of the DY-59 chitinase was more pronounced than that of the VS-9 chitinase, which is consistent with the fact that DY-59 chitinase activity was higher than that of the VS-9 chitinase.

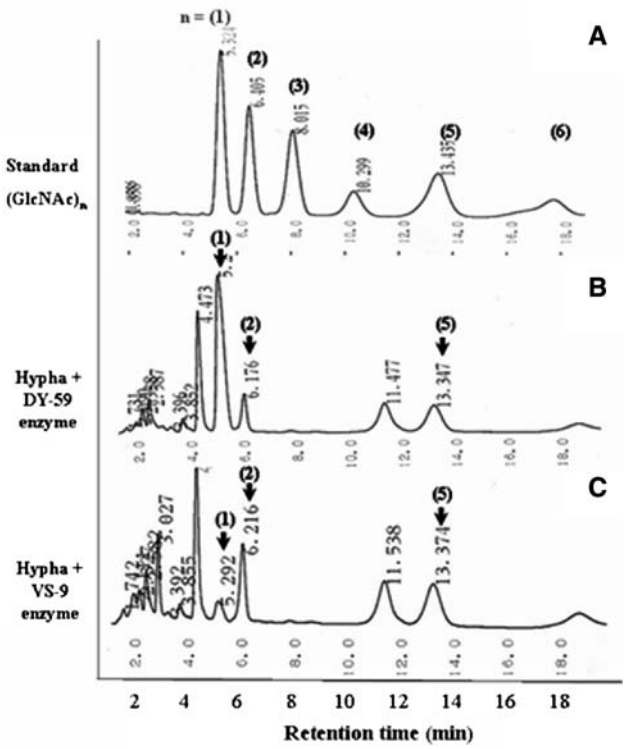

Fig. 3 Hydrolysis products from the cell wall of *F. solani* hypha by DY-59 and VS-9 crude chitinase. Chitin oligomer standard (1 ~ 6) **(A)**; hydrolysis products from DY-59 **(B)** and from VS-9 chitinases **(C)**

Hydrolysis by the crude enzymes of *F. solani* hypha as the substrate was verified by high-pressure liquid chromatography (HPLC)–based analysis. Six oligosaccharides were shown on a separation diagram of substances (Fig. 3). The monomer, dimer, and pentamer of chitin were determined by retention time via HPLC using external and internal chitin oligomer standards. The other products may be belonged to chitosan or glucan oligomers. Hydrolysis product contained 492.4 μg mL^{-1} of chitin monomer and 91.0 μg mL^{-1} of chitin dimer by DY-59 chitinase, and 82.6 μg mL^{-1} of chitin monomer and 87.0 μg mL^{-1} of chitin dimer by VS-9 chitinase at 24-h incubation. The amount of monomer produced from the *F. solani* hypha by the DY-59 chitinase was much more than that created by the VS-9 chitinase. This result can be explained by the fact that *N*-acetyl-*β*-D-glucosaminidase activity of DY-59 strain was higher than that of the VS-9 strain. Compared with enzymatic hydrolysis of swollen chitin from crab shell, previously described, more chitin oligomers were shown, including dimer and pentamer for DY-59 chitinases and pentamer for VS-9 chitinases. It may be thought that chitin structure in fungal cell walls is different from crab swollen chitin.

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