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ORIGINAL ARTICLE

Purification and Characterization of a β-*N*-Acetylhexosaminidase from Wheat Bran and Its Applicability to Biocontrol of *Fusarium solani*

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Abstract *N*-acetyl-β-D-hexosaminidase was purified from wheat bran and characterized. The purified enzyme showed two protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular mass of 75 and 78 kDa. The enzyme exhibited optimum pH and temperature at 5.0 and 50°C, respectively. The enzyme was active on the substrates of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-GlcNAc) and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (*p*NP-GalNAc), whereas inactive on *p*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, swollen chitin, and colloidal chitin, suggesting high substrate specificity. The enzyme activity for *p*NP-GlcNAc was stable at pH 3–6 and under 50°C. The K_m, V_{max} and K_{cat} for *p*NP-GlcNAc were 0.014 mM, 0.05 μmol/min, and 3.01×10^6 min^{-1}, respectively. The enzyme could be completely inhibited at 1–10 mM $HgCl_2$ and $AgNO_3$, suggesting that the intact thiol group is essential for activity. β-*N*-Acetylhexosaminidase from wheat bran could inhibit the conidial germination and digest the hyphae of *Fusarium solani*.

Keywords biocontrol · *Fusarium solani* · *N*-acetyl-β-D-hexosaminidase · *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide · wheat bran

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Introduction

N-Acetylglucosamine (GlcNAc) is present as a component of the sugar chain of glycoproteins and glycolipids in plants (Oikawa et al., 2003). GlcNAc also constitutes signal molecules that function in the interaction of plants with microorganisms, such as *N*-acetyl-chitooligosaccaride elicitors for plant defense against pathogens (Ren and West, 1992; Yamada et al., 1993; Ishihara et al., 1998) and Nod factors that induce symbiotic reactions in fabaceous plants (Truchet et al., 1991). The degradation of those molecules that have GlcNAc residues is conducted by chitinolytic enzymes, including *N*-acetyl-β-D-hexosaminidase (β-HexNAc'ase, EC 3.2.1.52), chitinase (EC 3.2.1.14) and endo-*N*-acetyl-D-glucosaminidase (EC 3.2.1.96).

β-HexNAc'ase catalyzes the hydrolysis of terminal non-reducing *N*-acetyl-D-hexosamine residues in *N*-acetyl-β-D-hexosaminides, and thus, by definition, is an exo-glycosidase (Horsch et al., 1997). It has both activities of *N*-acetylglucosaminidase (GlcNAc'ase) and *N*-acetylgalactosaminidase (GalNAc'ase) (Oikawa et al., 2003). These enzymes have recently gained a lot of attention, not only due to their great potential in the enzymatic synthesis of carbohydrates (Nieder et al., 2004) but also due to their implication in human physiology and disease (Hart et al., 2007).

β-HexNAc'ases have been shown to be universally distributed among various types of living organisms, both prokaryotic and eukaryotic, excluding the kingdom of archaea. This enzyme has a very broad range of functions depending on the organism and on the localization in the animal or plant body or even within the cell. Plant β-HexNAc'ase activities have been characterized in fenugreek (Bouquelet and Spike, 1978), rice seeds (Jin et al., 2002), wheat leaf (Barber and Ride, 1989), cabbage (Chang et al., 1998), pea seeds (Harley and Beevers, 1987), and *Lupinus luteus* L. seeds (Pocsi et al., 1990). Even though a number of plant β-HexNAc'ases have been reported, the physiological role of this enzyme remains yet unclear (Horsch et al., 1997; Park et al., 2001b).

There are indications that β-HexNAc'ases are employed in seed germination, because increasing activity of β-HexNAc'ase was observed during the germination of rice and maize seeds, cleaving off the glycan fractions of storage glycoproteins (Harris and Chrispeels, 1975; Yi, 1981). Jagadeesh and coworkers (2004) found that the significant increase in the activity of β-HexNAc'ase together with other glycosidases may be implicated in tomato and pepper ripening.

Some plant β-HexNAc'ases also degrade chitin and chitin oligomers (Li and Li, 1970; Yi, 1981; Barber and Ride, 1989). Therefore, their participation in the process of chitin-elicited lignification has been suggested. Several fungi, such as *Clonostachys* and *Trichoderma* species, produce β-HexNAc'ases upon specific induction by chitin or fungal cell wall, thus serving as potentially useful biocontrol agents against economically important plant pathogens (Horsch et al., 1997; Brunner et al., 2003; Mamarabadi et al., 2009). However, no plant β-HexNAc'ase has been reported on biological control of pathogenic fungi. In the present study, we purified and characterized a β-HexNAc'ases from wheat bran. To the best of our knowledge, this is the first report on wheat bran β-HexNAc'ases and their antifungal activity against *Fusarium solani*.

Materials and Methods

Materials. Chitin and chitosan were purchased from KittoLife Co. (Korea). Chitin and chitosan oligosaccharides were purchased from Seikagaku Co. (Japan). All others used were of reagent grade. Wheat bran (particle size ≥60 mesh) was supplied from Woori Wheat Bran Co. (Korea).

Preparation of crude enzyme. The crude enzyme from wheat bran was extracted by pouring 2 L sodium acetate buffer (0.1 M, pH 5.0) into a 5-L bottle containing 1 kg wheat bran. The suspension was stirred at 4°C overnight. After centrifugation at 8,000 rpm for 40 min, the supernatant was collected and stored at 4°C for further experiments.

Enzyme assay. β-HexNAc'ases activity was assayed with *p*NP-GlcNAc as the standard substrate following the method described by Chang et al. (1998). The reaction was performed at 37°C for 15 min in a test tube containing 50 μL of enzyme solution, 50 μL of 5 mM *p*NP-GlcNAc, and 100 μL of 0.1 M sodium acetate buffer (pH 5.0). The reaction was terminated by the addition of 1 mL of 0.2 M Na_2CO_3. One unit of β-HexNAc'ase activity was defined as the amount of enzyme required to produce 1 μmol of *p*NP per min.

Purificatioin of β-*N*-acetylhexosaminidase. Crude enzyme solution was first treated with 60% ammonium sulfate overnight, and the precipitate formed was centrifuged at 8,000 rpm for 40 min. The precipitate was dissolved in a small volume of distilled water, and then dialyzed against distilled water overnight at 4°C. The dialysate was applied to DEAE-Sephadex column (2.5 cm × 70 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0), and the column was washed with two bed volumes of equilibrium buffer. The enzyme was eluted stepwise with 0.1–0.5 M NaCl in the same buffer. The fractions with β-HexNAc'ase activity were pooled and dialyzed against distilled water overnight at 4°C. The dialysate was then applied to CM-Sephadex column (2.5 cm × 70 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted stepwise with 0.1–0.5 M NaCl in the same buffer. The fractions showing β-HexNAc'ase activity were pooled and concentrated with polyethylene glycol 6000. The concentrated enzyme was then applied to Sephacryl S-300 column (1.7 cm × 45 cm), which was eluted with 20 mM sodium acetate buffer containing 0.15 M NaCl. The fractions containing β-HexNAc'ase activity were pooled and concentrated. The protein concentration during all purification steps was followed with a UV monitor at 280 nm.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Protein content was determined by the method described by Bradford (1976).

Characterization of purified β-*N*-acetylhexosaminidase. For pH effect, the enzyme activity was measured at pH 3.0–11.0 using *p*NP-GlcNAc or *p*NP-GalNAc as substrate. The pH stability was determined by pre-incubating the enzyme in buffer solutions of various pH values for 24 h at 4°C. Residual enzyme activity was measured, and the relative activity was calculated. The following buffer systems were used at 50 mM each acetate buffer for pH 3.0–7.0, phosphate buffer for pH 7.0–8.0, and carbonate buffer for pH 9.0–11.0. To determine the optimum temperature, the enzyme was incubated with the substrate of *p*NP-GlcNAc or *p*NP-GalNAc at various temperatures (20–70°C) at pH 5.0 for 30 min. Thermal stability was determined by incubating the enzyme at various temperatures for 2 h. Residual enzyme activity was measured, and the relative activity was calculated. To specify the effect of metal ions on enzyme activity, the enzyme reaction was performed under standard conditions in the presence of 1 or 10 mM various metal ions. Enzyme activity in the absence of any metal ion was considered as 100%. For substrate specificity, activity of the enzyme on several substrates was determined. The substrates tested were *p*NP-GlcNAc, *p*NP-GalNAc, *p*NP-β-glucopyranoside, *p*NP-β-galactopyranoside, glycol chitin, and swollen chitin. For kinetic constants, enzyme preparation was incubated with various concentrations of *p*NP-GlcNAc and *p*NP-GalNAc, between 2.0 and 10.0 mg/mL. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by Lineweaver-Burk transformation.

Inhibition of conidial germination of *F. solani*. *F. solani* spore suspension was harvested from PDA plates after cultivation at 25°C for 9 days. A solution containing 0.05% glucose and 0.05 % KH_2PO_4 was added on the surfaces of fungal colonies. After slight agitation, the spore suspension was collected. The number of conidia in the suspension was determined using a haemacytometer (Marienfeld, Germany) and adjusted to approximately 10^8 spores per mL. To estimate the inhibition effect of β-HexNAc'ase on conidial germination of *F. solani*, 0, 50, 100, and 150 μL of

enzyme preparations (5.5 U/mL) were added to 50 μL of *F. solani* spore suspention containing 10^8 spores per mL, and the final volume was adjusted to 200 μL using heated enzyme. The mixtures were incubated at 30°C for 4–20 h. After incubation, the number of germinated conidia was counted. The inhibition rate was estimated using the following equation:

$$\text{Inhibition rate (\%)} = (C - E)/C \times 100\%$$

where C is the number of germinated conidia in the control, and E is the number of germinated conidia in the enzyme-treated spore suspension. To determine the effect of β-HexNAc'ase on the lysis of the hyphae from *F. solani*, a mixture (total 200 μL) consisting of 50 μL germinated spore solution, and the enzyme solutions were incubated at 30°C. The digested cell walls of germinated tubes were observed and photographed after 8 and 20 h digestion of walls.

Results and Discussion

Purification of β-*N*-acetylhexosaminidase from wheat bran. β-HexNAc'ases have been detected in a wide variety of plants (Horsch et al., 1997; Park et al., 2001a), and most of these enzymes with high activities were detected from plant leaves and seeds (Barber and Ride, 1989; Jin et al., 2002). Herein, we describe the purification of β-HexNAc'ases from wheat bran. The enzyme was purified to about 18.3-fold as compared with the crude enzyme by 60% ammonium sulfate precipitation, DEAE-Sephadex chromatography, CM Sephadex chromatography, and Sephacryl S-300 chromatography (Table 1). Only one peak related to the activity of β-*N*-acetylhexosaminidase was shown on Sephacryl S-300 chromatography (Fig 1B). However, SDS-PAGE analysis of the purified enzyme showed two protein bands with a molecular weight of 75 kDa and 78 kDa (Fig. 2), which may be

Table 1 Purification of β-*N*-acetylhexosaminidase from wheat bran

Purification Step*	Total protein (mg)**	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude enzyme	529.7	12777.6	24.1	100	1.0
DEAE Sephadex	18.9	1225.1	64.6	9.5	2.7
CM Sephadex	11.7	861.6	73.6	6.7	3.1
Sephacryl S-300	0.7	311.3	444.7	2.4	18.3

*All operations were carried out at 4°C.
**Protein amounts were estimated using the Bradford method.

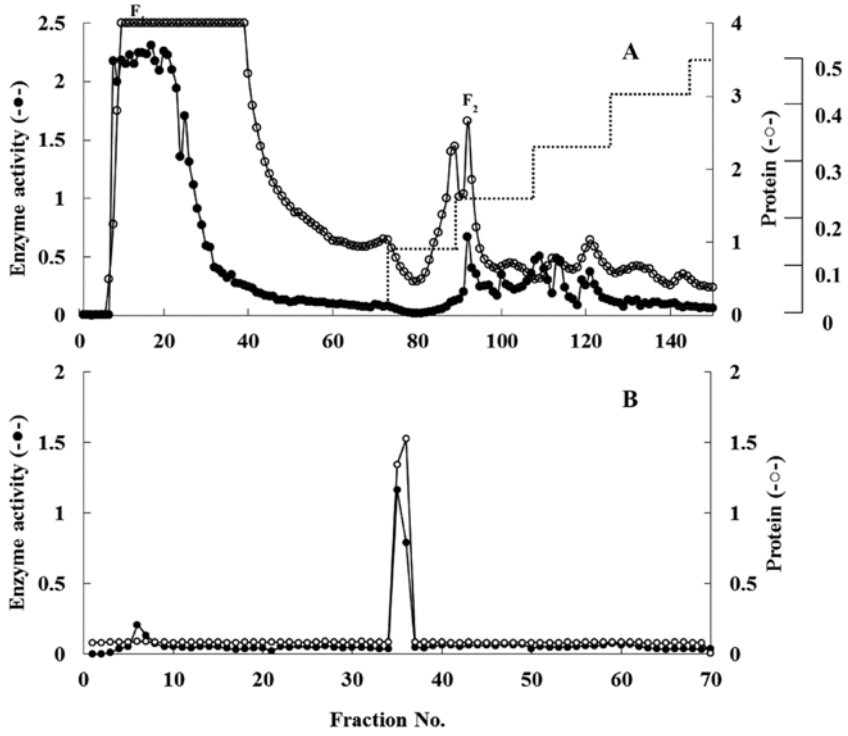

Fig. 1 Purification of β-*N*-acetylhexosaminidase from whean bran. Ion-exchange chromatography on DEAE-Sephadex column (A) and Gel-filtration chromatography on Sephacryl S-300 (B). Fraction F1 in Fig. 1A has been further purified at the next steps.

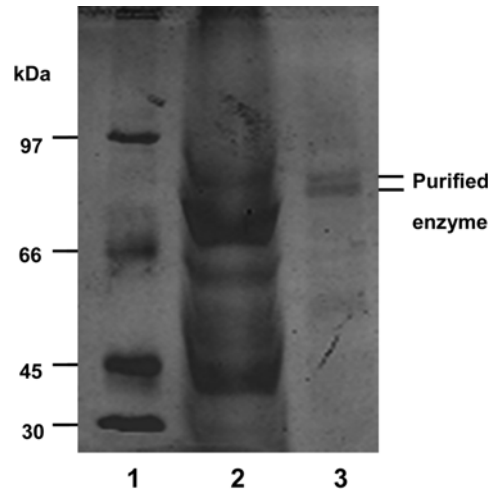

Fig. 2 SDS-PAGE of β-*N*-acetylhexosaminidase purified from wheat bran. Lane 1, protein markers; lane 2, crude enzyme; lane 3, purified enzyme.

due to isomerization of the enzyme. Actually, many plant β-HexNAc'ases were observed as isozymes. For instance, five major isozymes were identified from wheat leaf with the isoelectric points in the range 5.13–5.36 (Barber and Ride, 1989). Two isozymes with molecular weights of 45 and 90 kDa were resolved from mung bean seeds by gel filtration (Dey, 1984).

Effect of pH and temperature on β-*N*-acetylhexosaminidase activity. The optimum pH for the enzyme activity was between 4.0 and 6.0, and the enzyme was stable between pH values 3.0 and 6.0 (Fig. 3). The enzyme activity dramatically decreased at a pH over 6.0 and was completely lost at pH over 8.0. (Fig. 3B) at the individual pH. Jin et al. (2002) reported that the optimum pH of β-*N*-acetylhexosaminidase from rice seeds for hydrolysis of *p*NP-GlcNAc was higher than that for *p*NP-GalNAc. The optimum temperature for the enzyme activity was 50°C with both substrates of *p*NP-GlcNAc and *p*NP-GalNAc (Fig. 4). More than 80% of its activity was lost when the reaction temperature was higher than 60°C (Fig. 4). The profile of temperature stability towards *p*NP-GlcNAc and *p*NP-GalNAc was similar. The enzyme was stable at 20–50°C but unstable over 50°C with both substrates of *p*NP-GlcNAc and *p*NP-GalNAc (Fig. 4).

Substrate specificity of β-*N*-acetylhexosaminidase and kinetic parameters. β-HexNAc'ase from wheat bran released *p*-nitrophenol group from both *p*NP-GlcNAc and *p*NP-GalNAc (Table 2). However, it was virtually inactive towards *p*NP-β-glucopyranosaminide, *p*NP-β-galactopyranosaminide, swollen chitin, and glycol chitin, suggesting its high substrate specificity (Table 2). This result is consistent with previous reports. For instance, β-HexNAc'ases from wheat (Barber and Ride, 1989) and rice seeds (Jin et al., 2002) were also active towards both *p*NP-GlcNAc and *p*NP-GalNAc, but were unable to release GlcNAc from chitin. The relative activity of β-HexNAc'ase from wheat bran on substrate of *p*NP-GlcNAc was slightly higher than that on *p*NP-

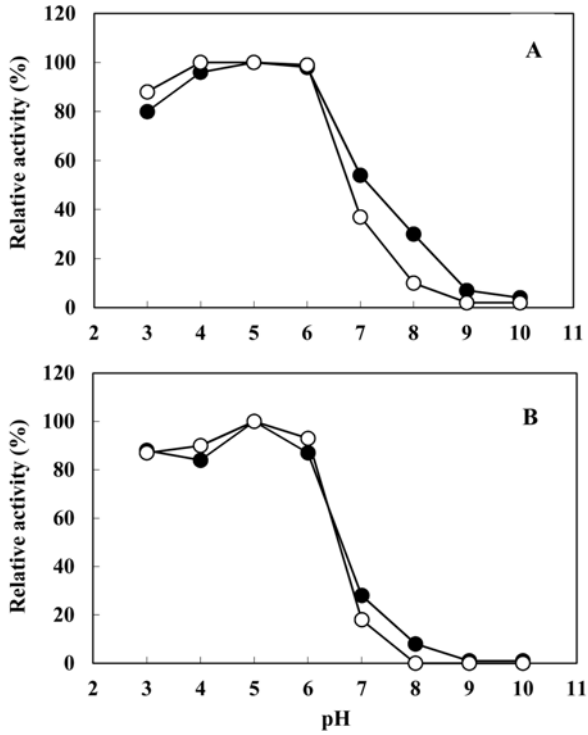

Fig. 3 Effect of pH on the activity of β-*N*-acetylhexosaminidase from wheat bran. Enzyme reactions were separately performed with the substrates of *p*NP-GlcNAc (A) and *p*NP-GalNAc (B). -●-, optimal pH; -○-, pH stability.

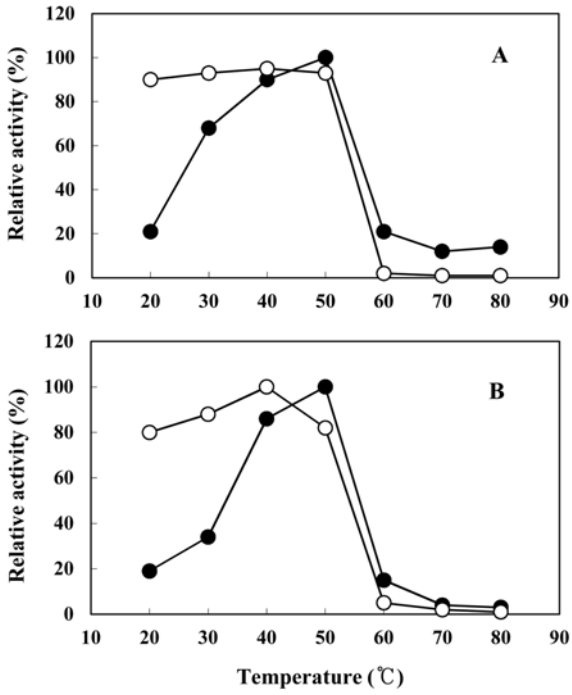

Fig. 4 Effect of temperature on the activity of β-*N*-acetylhexosaminidase from wheat bran. Enzyme reactions were separately performed with the substrates of *p*NP-GlcNAc (A) and *p*NP-GalNAc (B). -●-, optimal temperature; -○-, temperature stability.

Table 2 Substrate specificity of the β-*N*-acetylhexosaminidase from wheat bran

Substrate	Relative activity (%)
*p*NP-GlcNAc	100
*p*NP-GalNAc	90
*p*NP-β-glucopyranoside	0
*p*NP-β-galactopyranoside	0
Glycol chitin	0
Swollen chitin	0

GalNAc (Table 2). According to previous reports, plant β-HexNAc'ases generally exhibited different hydrolysis ratios towards *p*NP-GlcNAc and *p*NP-GalNAc, ranging from 0.7 to 143.0 (Horsch et al., 1997). It has been reported that the enzyme activity ratio on substrates of *p*NP-GlcNAc and *p*NP-GalNAc are pH-dependent due to different pH optimum for different substrates (Barber and Ride, 1989).

The kinetic parameters of the enzyme on substrates of *p*NP-GlcNAc and *p*NP-GalNAc were also measured and compared, based on Lineweaver-Burk plots. The K_m values on substrates of *p*NP-GlcNAc and *p*NP-GalNAc were established to be 0.014 and 0.03 mM, respectively (Table 3). To the present, K_m of β-HexNAc'ases from diverse plants for substrate of *p*NP-GlcNAc varied from 0.09 to 2.0 mM (Horsch et al., 1997). Therefore, wheat bran β-HexNAc'ases exhibited a lower K_m value than those of other plants. V*max* and K*cat* were also calculated (Table 3).

Effects of metal ions on β-*N*-acetylhexosaminidase activity. The effects of various metal ions on the enzyme activity are presented in Table 4. The enzyme activity was completely inhibited by 1–10 mM Hg^{2+} and also was strongly inhibited in the presence of 1–10 mM Ag^{+}. In addition, inhibition effects on enzyme activity were also observed with 1–10 mM of Al^{3+}, Sn^{2+}, Pb^{2+} Zn^{2+}, and Mg^{2+} (Table 4). These results suggested that the intact thiol group is essential for the activity, as seen in rice seeds (Jin et al., 2002) and wheat leaf (Barber and Ride, 1989). Small molecular inhibitors of β-HexNAc'ases have received a great deal of attention both as tools for elucidating the role of these enzymes in biological processes as well as for developing therapeutic interventions with minimal side effects (Horsch et al., 1997).

Inhibition of conidial germination of *F. solani*. β-HexNAc'ase from wheat bran exhibited inhibitory effects on spore germination of *F. solani* (Table 5). After a treatment of conidia with 50 μL of wheat bran β-*N*-acetylhexosaminidase (5.5 U/mL) for 8 h, more than 85% of germination inhibition of *F. solani* was observed. After 20 h treatment, most spores were not germinated (Table 5 and Fig. 5). In another experiment, when the germinated hyphae of *F. solani* were treated by enzyme solution, digestion of the hyphae of *F. solani* was observed (Fig. 6B). After 20 h treatment of the hyphae of *F. solani* with 50 μL of purified enzyme solution, a significant digestion of the hyphae was observed (Fig. 6C).

These could be anticipated from the suggestion that β-HexNAc'ases, as a component of the extracellular binary chitinolytic system effecting the complete degradation of chitin to GlcNAc, is a part of the enzyme inventory necessary for the heterolytic digestion of chitinous materials including chitin and fungal cell wall (Haran et al., 1995; Horsch et al., 1997). Previously, several

Table 4 Effect of metal ions on β-*N*-acetylhexosaminidase from wheat bran

Metal ions	Relative activity (%)	
	1 mM	10 mM
Control	100	100
Ag^{+}	26	4
K^{+}	100	100
Hg^{2+}	0	0
Mg^{2+}	92	90
Sn^{2+}	98	72
Cu^{2+}	100	96
Al^{3+}	98	74
Pb^{2+}	94	92
Zn^{2+}	92	89

Table 5 Inhibition of microconidial germination of *F. solani* by wheat bran β-*N*-acetylhexosaminidase

Amount of enzyme (μL)	Incubation time (h)			
	0	4	8	20
0	0.0±0.0	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}
50	0.0±0.0	16.2±7.2^{b}	85.5±3.0^{a}	97.5±4.2^{a}
100	0.0±0.0	29.8±6.1^{ab}	88.1±5.8^{a}	100.0±0.0^{a}
150	0.0±0.0	45.8±4.7^{a}	89.3±3.8^{a}	100.0±0.0^{a}

Enzyme activity of 5.5 U/mL was treated as described in Materials and Methods. Values in the vertical column followed by different superscripted letters are significantly different at $p \leq 0.05$ as determined using Tukey's HSD (Honestly Significant Difference) test.

Table 3 Kinetic parameters of β-*N*-acetylhexosaminidase from wheat bran

Parameters	Substrate		Ratio (*p*NP-GlcNAc'ase/*p*NP-GalNAc'ase)
	*p*NP-GlcNAc	*p*NP-GalNAc	
K_m (mM)	0.014	0.03	0.46
V_{max} (μmol/min/mg)	0.05	0.09	0.56
K_{cat} (min^{-1})	3.01×10^{6}	5.4×10^{6}	0.55
K_{cat}/K_m ($min^{-1}mM^{-1}$)	2.15×10^{8}	1.8×10^{8}	1.2
V_{max}/K_m (L/min)	3.57×10^{-3}	3.0×10^{-3}	1.2

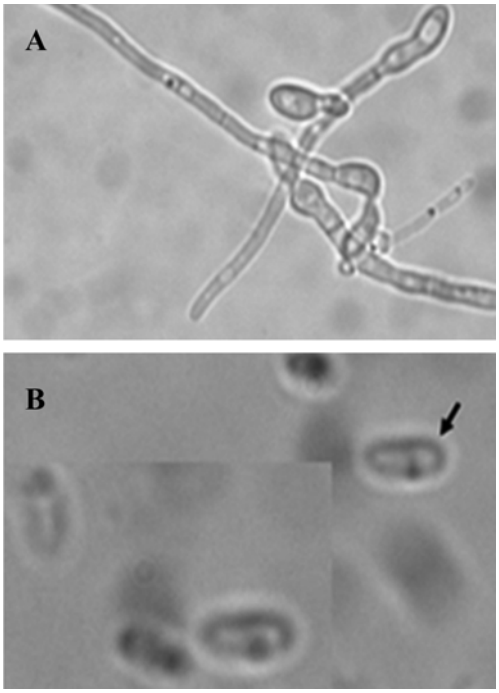

Fig. 5 Inhibition of microconidial germination of *F. solani* by β-*N*-acetylhexosaminidase from wheat bran. (A), Normal microconidial germination; (B), ungerminated conidia treated with purified β-HexNAc'ase. (1,000×).

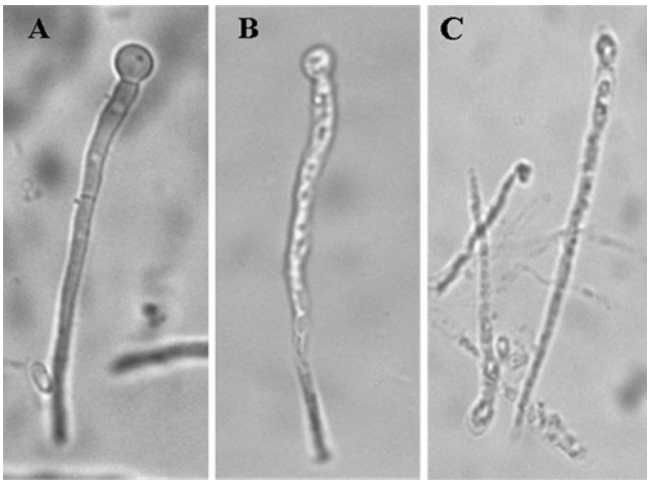

Fig. 6 Digestion of *F. solani* hypha by β-*N*-acetylhexosaminidase from wheat bran. (A), control; (B), hypha of *F. solani* was treated with purified β-HexNAc'ase for 8 h; (C), hypha of *F. solani* was treated with purified β-HexNAc'ase for 20 h. (1,000×).

fungal β-HexNAc'ases have been considered to play an essential role in the lysis of phytopathogenic fungal cell walls during their parasitic entrance into the plants, thus serving as potentially useful biocontrol agents against economically important plant pathogens (Horsch et al., 1997; Brunner et al., 2003; Mamarabadi et al., 2009). In *Candida albicans*, β-HexNAc'ase appears to be a virulence factor (Jenkinson and Shepherd, 1987). The production of extracellular β-HexNAc'ase is also a component of age-related autolysis of filamentous fungi (Pusztahelyi et al., 1997). The inhibition of conidial germination and the digestion of the fungal hyphae by wheat bran β-HexNAc'ases suggest its applicability for biocontrol of phytopathogenic fungi such as *Fusarium solani*. The significance and possible mode of action of plant β-HexNAc'ases in the inhibition of fungal germination and the digestion of the fungal hyphae need intensive studies in the future.

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