Chitinase-producing *Paenibacillus elgii* strain HOA73 has been used to control plant diseases. However, the antimicrobial activity of its extracellular chitinase has not been fully elucidated. The major extracellular chitinase gene (*PeChi68*) from strain HOA73 was cloned and expressed in *Escherichia coli* in this study. This gene had an open reading frame of 2,028 bp, encoding a protein of 675 amino acid residues containing a secretion signal peptide, a chitin-binding domain, two fibronectin type III domains, and a catalytic hydrolase domain. The chitinase (*PeChi68*) purified from recombinant *E. coli* exhibited a molecular mass of approximately 68 kDa on SDS-PAGE. Biochemical analysis indicated that optimum temperature for the activity of purified chitinase was 50°C. However, it was inactivated with time when it was incubated at 40°C and 50°C. Its optimum activity was found at pH 7, although its activity was stable when incubated between pH 3 and pH 11. Heavy metals inhibited this chitinase. This purified chitinase completely inhibited spore germination of two *Cladosporium* isolates and partially inhibited germination of *Botrytis cinerea* spores. However, it had no effect on the spores of a *Colletotricum* isolate. These results indicate that the extracellular chitinase produced by *P. elgii* HOA73 might have function in limiting spore germination of certain fungal pathogens.

**Keywords**: antifungal activity, *Botrytis cinerea*, extracellular chitinase, heterologous expression, *Paenibacillus elgii*

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Chitin, a β-1,4-linked linear biopolymer of *N*-Acetylglucosamine (NAG), is widely distributed in nature in the exoskeleton of arthropods, the outer shells of crustaceans and nematodes eggs, and fungal cell walls (Brzezinska et al., 2014). It is also present in the peritrophic matrix lining of the midgut of most invertebrates (Hegedus et al., 2009). Chitin is hydrolyzed by diverse chitinolytic enzymes with different modes of hydrolysis. These enzymes can be classified as endochitinases, exochitinases, chitobiases, and β-*N*-acetylglucosaminidases (Brzezinska et al., 2014). Chitinases can be produced by viruses, bacteria, fungi, insects, higher plants, and animals. These enzymes are involved in various biological processes, including morphogenesis, nutrition, and defense against pathogens contained in their cell walls (Yan and Fong, 2015). Chitinolytic enzymes have been commercially used to produce chitooligosaccharides and NAG as part of pharmaceutical formulations. Purified chitinase enzyme has been used to generate protoplasts from fungi and yeast, treat chitinous waste, control malaria transmission, and deter the growth of pathogenic fungi (Dahiya et al., 2006).

Chitinase-producing microbes are formulated as commercial biocontrol agents because they can lyse the cell walls of chitin-containing fungal pathogens (Flach et al., 1992; Kim et al., 2008, 2010, 2011). Chitinase is part of the biological control agents (Chet et al., 1990; Lorito et
al., 1994) produced by isolates of *Bacillus* spp. (Chang et al., 2003; Lee et al., 2009; Reyes-Ramírez et al., 2004) and fungal genera *Trichoderma* (Lorito et al., 1994). Chitinases produced by *Chromobacterium* sp. C61 and *Pseudomonas* sp. GRC3 have been used in the control of *Rhizoctonia solani* (Arora et al., 2007; Park et al., 2005). However, because plant disease suppression is correlated with multiple traits, effective agents used in each biocontrol system might vary (Kim et al., 2011).

Isolates of *Paenibacillus elgii* can inhibit the growth of human and plant pathogenic bacteria and fungi (Kim et al., 2016; Kumar et al., 2015). In addition, they can promote plant growth (Das et al., 2010) and induce systemic resistance in planta (Sang et al., 2014). The draft genome sequence of *P. elgii* B69 has revealed many genes associated with antibiotic synthesis (Ding et al., 2011), including genes for the synthesis of catecholate siderophores (paenibactin), lantibiotics (elgicins), antibiotic pelgit, and chitinases (Qian et al., 2012; Teng et al., 2012; Wen et al., 2011). It has been reported that *P. elgii* HOA73 is effective against root knot nematode, diamond back moth, and *Botrytis cinerea* (Neung et al., 2014; Nguyen et al., 2013, 2015). In previous work, protocatechueic acid isolated from *P. elgii* HOA71 has been identified as the key antifungal compound against *B. cinerea* (Nguyen et al., 2015). However, the role of extracellular chitinase in the antifungal activity of *P. elgii* HOA73 has not been reported yet.

Therefore, the objective of this study was to determine the properties of a major extracellular chitinase from biocontrol strain *P. elgii* HOA73. The gene encoding a major chitinase from isolate HOA73 was expressed in *Escherichia coli* to determine its biochemical properties and its potential antifungal activity against some plant pathogens. Our results showed that the production of extracellular chitinase by *P. elgii* HOA73 might play an important role in inhibiting spor germination of certain fungal pathogens.

Materials and Methods

**Bacterial strains and growth conditions.** The chitinase-producing strain *P. elgii* HOA73 was isolated from field soil under tomato cultivation in Korea (Neung et al., 2014). The strain was stored at −70°C as 20% glycerol stock. It was cultured in tryptic soy broth (TSB; Difco, Sparks, MD, USA). Strain HOA73 was deposited at Korean Agricultural Culture Collection (KACC; Wanju, Korea) under strain number KACC19018.

**Cloning and sequence analysis of a chitinase gene.** Genomic DNA was isolated from *P. elgii* HOA73 using PureHelix™ genomic DNA Prep kit (NanoHelix, Daejeon, Korea) and used as the template for PCR amplification. The gene encoding an extracellular chitinase was amplified using the following primers: Chi68F (5′-CGA CGA TGA TAT TAG CCG GA-3′) and Chi68R (5′-ACC CTT CGC TAC AGG ACA AA-3′). They were designed based on chitinase gene of *P. elgii* B69 (Ding et al., 2011). PCR reaction was performed with a HelixAmp™ Premium-Taq polymerase kit (NanoHelix). PCR products were cleaned using QIAquick columns (Qiagen, Valencia, CA, USA) and cloned with pGEM-T Easy kit (Promega, Madison, WI, USA). Recombinant plasmids containing PCR inserts were isolated using mini-plasmid purification system (Bioneer, Daejeon, Korea). PCR inserts were then sequenced using dye terminator on an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Solgent Company (Daejeon, Korea).

Signal peptides of the chitinase was predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0). The molecular weight and pl value of mature protein of chitinase were calculated using Compute pl/Mw in ExPASy (http://expasy.org/tools). Putative conserved domains were detected using protein blast program of National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Predicted domains were identified with SMART program (http://smart.embl-heidelberg.de) and further analyzed with CDSearch of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Homology for chitin-binding and catalytic domains were examined using pBLAST. Alignments were made compared to chitinase A1 from *Bacillus circulans* W-12. The chitinase gene sequence from *P. elgii* HOA73 was deposited at GenBank under accession number KX602288.

Expression and purification of the chitinase in *E. coli*. FastDigest Fermentas restriction enzymes (Thermo Fisher Scientific, Waltham, MA, USA) were used for gene cloning. Coding region of the chitinase gene without sequence of signal peptide was amplified by PCR using primer Chi68FN containing NdeI site (5′-GAA TTC CAT ATG ATG AAA CGA AAA GCT TG-3′) and primer Chi68RN containing XhoI site (5′-CCG CTC GAG CTA ATT CAG ACC GTT TTT C-3′). The PCR product was purified using gel extraction kit (Bioneer), digested with restriction enzymes *NdeI* and *XhoI*, and then ligated into pET-23b (+) vector (Novagen, Madison, WI, USA) followed by digestion with the same enzymes. These recombinant vectors were transformed into *E. coli* BL21 (DE3; Agilent Technologies, Santa Clara, CA, USA) for protein expression. Positive clones bearing the gene were identified by PCR after culturing cells in Luria-Bertani (LB)
medium containing ampicillin with shaking (200 rpm) at 37°C. When OD_{600 nm} reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4 mM) was added to the culture followed by incubation at 20°C for 14 h. Cells from 2 l broth were harvested by centrifugation at 8,000 rpm for 5 min, resuspended in ice cold 20 mM Tris-buffer (pH 8), ultrasonicated, and centrifuged at 8,000 rpm for 5 min.

Proteins in the supernatant were further purified by loading onto a Ni-NTA agarose column (Invitrogen, Santa Cruz, CA, USA) pre-equilibrated with binding buffer (20 mM Tris-HCl buffer at pH 8, 10 mM NaCl, and 20 mM imidazole). Weakly-bound impurities were washed out using washing buffer (20 mM Tris-HCl buffer pH 8, 10 mM NaCl, and 40 mM imidazole) at 10-times of the column volume. To elute proteins, a step-wise imidazole gradient (100 mM, 250 mM, and 500 mM imidazole) in 20 mM Tris-HCl buffer (pH 8) and 10 mM NaCl was used. After checking the sizes of eluted samples on 15% SDS-PAGE gels, eluates containing target protein were purified after loading onto DEAE SepharoseTM Fast Flow column (Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with binding buffer (20 mM Tris-HCl buffer pH 8, 10 mM NaCl, and 1 mM EDTA). Proteins were eluted with a gradient of NaCl (100 mM to 400 mM). The eluates containing target protein were concentrated using Centricon Ultracel PL-10 filter (Merck Millipore, Darmstadt, Germany) by centrifugation at 3,000 rpm for 10 min.

**Enzyme activity assay.** Enzyme activity assays were performed using various polysaccharides and synthetic substrates. Substrates included purified chitin (Sigma-Aldrich), colloidal chitin prepared from native chitin (Sigma-Aldrich), ethylene glycol chitin (Seikagaku, Tokyo, Japan), glycol chitosan (Sigma-Aldrich), cellulose, Avicel (Sigma-Aldrich), β-1,3 glucan, and laminarin (Sigma-Aldrich). Mixtures (0.5 ml) containing 0.5% (w/v) polysaccharides and 5 μl enzyme (1 mg/ml) in 0.5 M sodium phosphate buffer (pH 7) were incubated at 37°C for 1 h followed by boiling for 5 min. After centrifugation, 200 μl of supernatant solution was mixed with 260 μl of color reagent (0.05% potassium ferricyanide in 0.5 M Na₂CO₃) and boiled for 15 min. After cooling down, 100 μl of the reaction mixture was transferred to a 96-well microplate and the absorbance value was measured at wavelength of 420 nm on a BioTek uQuantTM (BioTek, Winooski, VT, USA). The amount of reducing sugar released from the reaction mixture was estimated using 0.16 to 2.5 mM N-acetylglucosamine (NAG) as standard. One unit of enzyme activity was expressed as micromole of liberated NAG per min per mg of purified chitinase protein.

The following synthetic chitin-based substrates were used 4-methylumbelliferyl N-acetyl-β-D-glucosamine (4-MU-GlcNAc), 4-methylumbelliferyl β-D-β, N', N'-diacetylchitobioside (4-MU-[β-GlcNAc]₂), and 4-methylumbelliferyl β-D-β, N', N'-triacyctchitotrioside (4-MU-[GlcNAc]₃). They were purchased from Sigma-Aldrich. The reaction mixture (0.1 ml) containing 0.5 mM synthetic substrate and 1 μl of enzyme (1 mg/ml) in 0.5 M sodium phosphate buffer (pH 7) was incubated at 37°C for 30 min and measured with excitation at 360 nm and emission at 440 nm on a BioTek FLX-8000 (BioTek). The amount of 4-MU released from the reaction mixture was estimated using 0.002 to 0.125 mM of 4-methylumbelliferone (Sigma-Aldrich) as a standard. One unit of enzyme activity was expressed as micromole of liberated 4-MU per min per mg of the purified chitinase protein.

**Chitinase PAGE analysis.** Proteins resolved on SDS-PAGE gels were silver stained and their molecular weights were determined by comparing to PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) (Park et al., 2007). Activities of the purified enzyme prepared from recombinant *E. coli* and culture supernatant of *P. elgii* HOA73 were compared to each other using 4-MU-(GlcNAc)₂ as substrate. The culture supernatant from *P. elgii* HOA73 was further analyzed on a gel containing glycol chitin to provide greater sensitivity than what was observed with substrate 4-MU-(GlcNAc)₂.

**Biochemical characterization of the purified enzyme.** Effects of temperature, pH, and metal ions on the activity and stability of the purified chitinase were determined using 4-MU-(GlcNAc), as substrate. Different temperatures (20–70°C) were tested. Thermal stability of purified enzyme was evaluated with standard assay after pre-incubating the enzyme in 500 mM phosphate buffer (pH 7) at indicated temperatures with various time intervals.

The optimal pH was determined in 50 mM buffer with pH ranging from 2 to 13. The following buffers were used glycine-HCl (pH 2–3), phosphate-citrate (pH 3–6), phosphate buffer (pH 6–7), Tris-HCl (pH 8–9), KCl-H₂BO₃-NaOH (pH 9–10), NaHCO₃-NaOH (pH 10–11), and KCl-NaOH (pH 12–13). To determine pH stability, the enzyme was incubated in the above-mentioned buffers at 4°C for various time intervals. After incubation, 500 mM of phosphate buffer (pH 7) with substrate 4-MU-(GlcNAc)_2 was added to measure the activity of enzyme.

Effect of metal ions, a metal chelator, and detergents on chitinase activity were determined by using the following: 5 mM of FeSO₄, ZnSO₄, CuSO₄, CaCl₂, MgSO₄, MnSO₄, CoCl₂, CoSO₄, HgCl₂, AgNO₃, KCl, LiCl, 2-mercaptoethanol, EDTA, SDS, 5% of Tween 20, Tween 80, or Triton X-100.
Antifungal activity assay. The purified enzyme was tested for its potential to inhibit conidial germination of plant pathogens. Pathogenic fungal isolates were obtained from KACC. Because *P. elgii* HOA73 was obtained from soil of tomato field, pathogens examined included the cause of tomato gray mold, *B. cinerea* 40574, and tomato leaf mold, *Fulvia fulva* 47762. Because *F. fulva* is related to *Cladosporium*, two other *Cladosporium* isolates causing post harvest problems, *C. sphaerospermum* 42600 and *C. tenuissimum* 46651, isolated from skin sooty dapple symptoms on pear fruits were used. Another pathogen, *Colletotrichum gloeosporioides* 40690, causative agent of red-pepper anthracnose, was also used.

Conidia were produced from these fungi by incubation at 25°C on potato-dextrose agar (PDA; Difco) and suspended in distilled water. The suspension was filtered through sterile Kimwipes to remove hyphal fragments and adjusted to 10^4 conidia/ml using 1/2 strength of potato-dextrose broth (pH 7; Difco). Reaction mixture (20 μl) containing the conidial suspension and 500 mM phosphate buffer (pH 7) was used as negative control. Spore germination was determined under an inverted microscope (Leica Microsystems, Wetzlar, Germany) after 8 and 24 h of incubation.

Fig. 1. Nucleotide and deduced amino acid sequence of *PeChi68*. The deduced amino acid sequence is given below the nucleotide sequence. Vertical arrow points at the cleavage site of the signal peptide. The black highlight shows putative ChtBD3 (aa 27–73). The gray highlights showed fibrinogen-binding domains, Fn3-1 (aa 83–156) and Fn3-2 (aa 177–250). The CaTD of Glyco_18 (aa 270–660) is in parentheses and its β-strands are in bold and underlined. The chitinase gene sequence from *Paenibacillus elgii* HOA73 is deposited at GenBank database under accession number KX602288.
tion with at least 100 spore/experiment. Spores producing germ tubes longer than their diameters were considered as germinated ones.

**Statistical analysis.** Data were analyzed through ANOVA using IBM SPSS Statistics version 21 (IBM Co., Armonk, NY, USA). If $F$ test was significant, differences were further elucidated through Duncan’s multiple range test. Different letters indicate significant different at $P < 0.05$. All experiments were repeated at least three independent times. Results are expressed as mean and standard deviation (SD).

**Results**

Sequence analysis of a chitinase gene from *P. elgii* HOA73. The chitinase gene designated as PeChi68 had an open reading frame (ORF) of 2,028 bp encoding 675 amino acid residues (Fig. 1). A putative N-terminus signal sequence allowing secretion of the enzyme had 26 amino acids with a cleavage site after Ala-26. Deduced mature protein (648 aa) had a molecular weight of 68,485 Da and a pI value of 5.94. Its mature protein was predicted to have two fibronectin type III domains (Fn3-1, aa 83–156; Fn3-2, aa 177–250), and a catalytic domain (CaTD) of glycosyl hydrolase family 18 (Glyco_18, aa 270–660).

Amino acid sequences of PeChi68 shared 27%, 29%, and 62% sequence identities with those of ChBDChiB, ChBDChiC, and ChBDChiA1, respectively. However, they shared higher sequence identities (> 70–98%) with chitinases of spore-forming bacteria, including endospore-forming *Paenibacillus* spp., *Brevibacillus* spp., and *Clostridium* spp. (Fig. 2).

PeChi68 was classified into subfamily A because the CaTD of PeChi68 shared 31% to 69% sequence identities with representative proteins belonging to subfamily A. However, it only shared 17% to 20% sequence identities with representative enzymes belonging to subfamily B (Fig. 3).

Secretion and molecular mass of PeChi68 expressed in *E. coli*. A single band with chitinase activity for substrate 4-MU-(GlcNAc)_2 was observed after SDS-PAGE electrophoresis of the purified enzyme expressed in *E. coli* BL21 (DE3) cells. These cells harbored vector pET-23b (+) bearing an insert of the coding region of *PeChi68* without the sequence of the signal peptide.

The molecular weight of band was about 68 kDa, similar to the band when supernatant from the culture medium of *P. elgii* HOA73 was assayed using the same substrate (Fig. 4). This molecular weight agreed well with the predicted size using gene sequence. SDS-PAGE analysis also indicated that the chitinase activity resided in a monomeric protein. SDS-PAGE analysis for the culture supernatant using glycol chitin as substrate also showed a band at size

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**Fig. 2.** Sequence alignment of chitin-binding domain type 3 (ChtBD3) of PeChi68 and other bacterial chitinases. Sequences of representative proteins of three subfamilies belonging to ChtBD3 (upper part) and domains exhibiting more than 70% identities with those of PeChi68 (bottom part) were aligned on the basis of three-dimensional structure of ChBDChiA1. Identity (Id) indicates percentage of sequences identical to those of ChtBD3 of PeChi68. Numbers at the left and right of each sequence represent the first and last residue positions in ChtBD3. Gray background shows amino acids well conserved in all three families. Black background shows amino residues suggested to play important roles in chitin binding activity. Amino acid sequences of PeChi68 are highlighted by dotted square.
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of 68 kDa with additional activity bands, suggesting that other chitinases were produced by P. elgii HOA73.

Substrate specificities and hydrolysis properties of the purified PeChi68. The purified chitinase could hydrolyze chitin powder, colloidal chitin, and ethylene glycol chitin, but not glycol chitosan. Its activity was the highest for ethylene glycol chitin, followed by chitin powder and colloidal chitin (Table 1). It had no activity for cellulose, avicel, β 1-3 glucan, or laminarin. These results indicate that PeChi68 could only digest glycosidic bonds between NAG residues.

To clearly understand the hydrolysis properties of PeChi68 for chitin oligomers, synthetic substrates of 4-MU-(GlcNAc)n were used (Table 1). No activity was found for 4-MU-(GlcNAc)3, indicating that PeChi68 is not

Table 1. Specificities of the purified PeChi68 using various polysaccharides* and 4-MU-(GlcNAc)n† as substrates

| Substrate                  | Specific activity (µM/min/mg)‡ | Relative activity (%)§ |
|----------------------------|------------------------------|------------------------|
| Powder chitin              | 2,207 ± 129 y                | 67                     |
| Colloidal chitin           | 1,608 ± 117 z                | 49                     |
| Glycol chitin              | 3,250 ± 124 x                | 100                    |
| Glycol chitosan            | 0                            | -                      |
| Avicel                     | 0                            | -                      |
| Laminarin                  | 0                            | -                      |
| 4-MU-(GlcNAc)1             | 0                            | -                      |
| 4-MU-(GlcNAc)2             | 3,113 ± 373 b                | 54                     |
| 4-MU-(GlcNAc)3             | 5,765 ± 853 a                | 100                    |

*Activity with polysaccharides was measured after incubation at 37°C at pH 7 for 60 min. Specific activity indicates micromoles of liberated N-Acetylglucosamine per min per mg of the purified protein.
†Activity for 4-methylumbelliferyl N-acetyl-β-D-glucosamine (4-MU-[GlcNAc]n) was measured after incubation at 37°C and pH 7 for 30 min. The specific activity indicates micromoles of liberated 4-MU per min per mg of the purified protein.
‡Data are presented as means ± standard deviations from three independent experiments. Zero (0) indicates that no product was generated in reaction mixture with the substrate. Different letters indicate significantly (P < 0.01) different levels of enzyme activity based on Duncan’s multiple range test.
§Relative activity (%) of the purified protein was represented in two groups, such as polysaccharides and 4-MU-(GlcNAc)n.
a N,N'-diacetylchitobiase (chitobiase). When 4-MU-(GlcNAc)_2 and 4-MU-(GlcNAc)_3 were used as substrates, 4-MU was released. PeChi68 predominantly produced (GlcNAc)_2 from powdered and colloidal chitin, indicating that the enzyme had both endochitinase and exo-N,N'-diacetylchitobiohydrolase activities.

**Effect of temperature, pH, and metal ions on the activity of PeChi68.** The purified PeChi68 showed increasing activities at temperature above 20°C, with maximum activity found at 50°C. However, it was inactivated at 60°C (Fig. 5A). After incubation for 30 min, PeChi68 was found to be stable at temperatures below 50°C. However, its stability was decreased with increasing incubation time.

**Fig. 5.** Effect of temperature on the activity (A) and stability (B) of purified PeChi68. The activity was measured at 37°C at pH 7 using 4-MU-(GlcNAc)_2 as substrate. Relative activity (A) was calculated as percentage of activity value at defined temperature (20°C, 30°C, 40°C, or 50°C) compared to the highest activity value. The stability (B) was determined by measuring the residual activity after incubation at defined temperature (20°C, 30°C, 40°C, or 50°C) at pH 7 with 500 mM sodium phosphate buffer for given time period without substrate. Residual activity is the percentage of activity value compared to the highest activity value. Data are presented as means from three independent experiments. Error bars indicate standard deviations. Different letters indicate significantly ($P < 0.01$) different levels of enzyme activity based on Duncan’s multiple range test.

**Fig. 6.** Effect of pH on activity (A) and stability (B) of the purified PeChi68. The activity was measured at 37°C using 4-MU-(GlcNAc)_2 as substrate within pH range of 2 to 13 in 50 mM buffers with defined pH ranges, including glycine-HCl (G-H, pH 2–3), phosphate-citrate (P-C, pH 3–6), phosphate buffer (P buffer, pH 6–7), Tris-HCl (T-H, pH 8–9), KCl-H_2BO_3-NaOH (K-H-N, pH 9–10), NaHCO_3-NaOH (N-N, pH 10–11), and KCl-NaOH (K-N, pH 12–13). The relative activity is the percentage of activity value compared to the highest activity value. The stability was determined by measuring the residual activity at 37°C and pH 7 after incubation at various pH and 4°C for given time period without substrate. Residual activity is the percentage of activity value compared to the highest activity value at pH 7. Data are presented as means from three independent experiments. Error bars indicate standard deviations.
at higher temperatures. After incubation for 24 h, the remaining activities of PeChi68 were approximately 88% at 20°C or 30°C, 45% at 40°C, and 22% at 50°C (Fig. 5B).

PeChi68 was most active at pH 7 (Fig. 6A). Buffers showed effects on enzyme activity. For instance, the enzyme was much more active in glycine-HCl buffer than in phosphate-citrate buffer at pH 3. Its activity in phosphate buffer was higher than that in phosphate-citrate buffer at pH 6 (Fig. 6A). Its pH stability was also better in glycine-HCl buffer than that in phosphate-citrate buffers. For example, its activity in glycine-HCl at pH 3 was higher than that in phosphate-citrate at pH 4. Its activity in NaHCO₃-NaOH at pH 10 or pH 11 was also higher than that in other buffers at pH 4 to pH 10. Residual activity after incubation for 24 h was decreased in buffers at pH 3 to pH 6, but slightly increased in buffers at pH 8 to pH 10 with an incubation time of 2 h. At pH 12, residual activity was about 48% after incubation for 2 h. However, its activity was undetectable after incubation for 24 h. Therefore, the enzyme displayed stability at pH 3 to pH 11 (Fig. 6B).

The activity of PeChi68 was sensitive to heavy metals. Its activity was completely inhibited by 5 mM of HgCl₂, AgNO₃, and CoCl₂. Its activity was partially inhibited by 5 mM of CuSO₄, CoSO₄, and ZnSO₄. Treatments with 5 mM of FeSO₄, CaCl₂, MgSO₄, MnSO₄, KCl, or LiCl had no effect on its activity. Reducing reagent mercaptoethanol had no effect on its activity. Surfactants SDS, Triton X-100, Tween 20, and Tween 80 had little to no effect on its activity (Table 2). EDTA had no effect on its activity either (Table 2).

**Table 2.** Effects of various chemical reagents on the activity of the purified PeChi68*

| Chemical reagents (5 mM) | Specific activity (µM/µg)† | Relative activity (%) |
|-------------------------|---------------------------|----------------------|
| Control                 | 11.8 ± 0.7 ab             | 100                  |
| FeSO₄                   | 12.3 ± 0.6 a              | 104                  |
| LiCl                    | 11.6 ± 0.5 a              | 98                   |
| KCl                     | 11.4 ± 0.9 a              | 97                   |
| 2-Mercaptoethanol        | 11.3 ± 1.2 ab             | 96                   |
| MgSO₄                   | 11.2 ± 0.7 a              | 95                   |
| Tween 20                | 10.8 ± 1.2 ab             | 92                   |
| MnSO₄                   | 10.6 ± 1.0 ab             | 90                   |
| Tween 80                | 10.5 ± 1.0 ab             | 89                   |
| Triton X-100            | 10.3 ± 0.8 ab             | 87                   |
| EDTA                    | 10.0 ± 0.7 b              | 85                   |
| CaCl₂                   | 9.8 ± 1.5 bc              | 82                   |
| ZnSO₄                   | 7.6 ± 0.6 c               | 64                   |
| SDS                     | 5.2 ± 0.8 d               | 43                   |
| CoSO₄                   | 5.1 ± 0.6 d               | 43                   |
| CuSO₄                   | 3.5 ± 0.6 d               | 30                   |
| CoCl₂                   | 0.3 ± 0.1 d               | 3                    |
| HgCl₂                   | 0 e                       | 0                    |
| AgNO₃                   | 0 e                       | 0                    |

*The purified PeChi68 (5 µg) was incubated with various reagents (5 mM) at 37°C, pH 7.0 for 30 min and residual chitinase activities were measured. No addition was made to the reaction mixture to determine the activity of the enzyme under control condition. Chitinase activity was defined as micromoles of liberated 4-methylumbelliferone per 1 µg of the purified PeChi68 for 1 min at 37°C.

†Data are presented as mean ± standard deviation from three independent experiments. Different letters indicate chitinase activity of the purified PeChi68 upon incubation with designated chemicals was significantly (P < 0.05) different from that of the control according to Duncan’s multiple range test.

**Antifungal activity of PeChi68 against phytopathogenic fungi.** The purified enzyme PeChi68 showed different degrees of inhibition on spore germination of two tomato pathogens. It had little effect on *F. fulva*. However, it inhibited spore germination for spores of *B. cinerea* in a dose-dependent manner. Spore germination was completely inhibited by low concentration of PeChi68 (100 µg/ml) for two *Cladosporium* isolates. However, germination of *Colletotrichum* spores was not inhibited by PeChi68 even at high doses (over 500 µg/ml) (Fig. 7).

![](https://via.placeholder.com/150)

**Fig. 7.** Inhibitory effect of purified PeChi68 on conidial germination of different plant pathogenic fungi: *Cladosporium sphaerospermum* (Cs), *Cladosporium tenuissimum* (Ct), *Botrytis cinerea* (Bc), *Fulvia fulva* (Ff), and *Colletotrichum gloeosporioides* (Cg). Data are presented as means from three independent experiments. Error bars indicate standard deviations. Spore germination was determined by light microscopy after 24 h of incubation with at least 100 spore/experiment. Spores producing germ tubes longer than their diameter were considered as germinated ones. Data are presented as mean values ± standard error from triplicates of three independent experiments.
Discussion

Using the SMART program, the mature protein of PeChi68 was predicted to have a ChtBD3, two Fn3 and a CaTD of Glyco_18 (Fig. 1). The ChtBD3 family is divided into three subfamilies: CBD_like, ChiC_BD, and ChiA1_BD. Their representative proteins in the NCBI database are ChBDChiB, ChBDChiC, and ChBDChiA1, respectively. Akagi et al. (2006) have suggested that Trp479 and Tyr481 in ChBDChiB, Trp59 and Trp60 in ChBDChiC, and Trp687 in ChBDChiA1 are important to distinguish different family members. Based on such criteria, PeChi68 was more similar to ChBDChiA1.

SmartBLAST of NCBI revealed that amino acid sequences and domains of PeChi68 were similar to those of chitinases of another P. elgii isolate (WP 063180230.1). They were also similar to Paenibacillus sp. MS11 (WP_036684856.1) and P. ehimensis (WP_025649605.1). Domains detected in PeChi68 were also similar to those of chitinase A1 from B. circulans WL-12 (Hashimoto et al., 2000).

NCBI sequence homology search revealed that PeChi68 chitinase belonged to ChiA1_BD sub-family of ChtBD3. The representative member of ChiA1_BD sub-family is ChBD of chitinase A1 (ChiA1) from B. circulans WL-12 that can bind to insoluble chitin (Hashimoto et al., 2000; Watanabe et al., 1994). The CaTD of PeChi68 was classified as a glycosyl hydrolase family 18 type II chitinase (GH 18 chitinase) containing ChiA1 from B. circulans WI-12 and chitinase A from Serratia marcescens. These chitinases have high hydrolyzing activity against insoluble chitin (Suzuki et al., 2002; Watanabe et al., 1990). It has been reported that the Fn3 domain of ChiA1 from B. circulans WI-12 plays an important role in the hydrolysis of chitin, although it is not directly involved in chitin binding (Watanabe et al., 1994).

A chitinase (ChiE) from Paenibacillus sp. strain FPU-7 with high activity against pNP-(GlcNAc)_2 has been reported (Itoh et al., 2013). This strain and P. elgii strain 69 have multiple chitinolytic enzyme genes in their genomes. Therefore, SDS-PAGE analysis of extracellular proteins of P. elgii strain HOA73 should have an array of genes encoding chitinolytic enzymes. However, only one chitinase (PeChi68) with activity for substrate 4-MU-(GlcNAc)₂ was detected from P. elgii HOA73 under our experimental conditions. Hydrolyzing properties of PeChi68 were similar to those of chitinase A (ChiA) from B. circulans WL-12 and chitinase A (ChiA) from S. marcescens (Brurberg et al., 1996; Horn et al., 2006; Suzuki et al., 2002; Watanabe et al., 1990). It has been suggested that ChiA has both exo-N,N′-diacetylchitobiohydrolase activity and endochitinase activity (Brurberg et al., 1996).

PeChi68 was slightly more active for 4-MU-(GlcNAc)₂ than for 4-MU-(GlcNAc)₃ (Table 1), similar to ChiA1 (Watanabe et al., 1990). However, ChiA showed similar activities for both substrates (4-MU-[GlcNAc]₃, and 4-MU-[GlcNAc]₄) (Brurberg et al., 1996). The hydrolysis potential of chitinase for artificial 4-MU derivatives might differ from that for native chitin (Brurberg et al., 1996; Watanabe et al., 1990).

Our results showed that chitinase PeChi68 showed various inhibitory activities against conidial germination depending on the species of fungus, in agreement with previous findings showing that bacterial and fungal chitinases have various inhibitory activities against spore germination depending on the source of spores (Banani et al., 2015; Broadway et al., 1995; Di Maro et al., 2010; Frankowski et al., 2001; Harman et al., 1993; Kamensky et al., 2003; Yu et al., 2015). It has been reported that one chitinase produced by a yeast can completely inhibit conidial germination of Monilinia spp., a postharvest pathogen causing of brown rot of stone fruits (Banani et al., 2015). Chitinase PeChi68 from P. elgii strain HOA73 also inhibited conidial germination of two other postharvest fruit pathogens, C. sphaerospermum and C. tenuissimum, raising the possibility that P. elgii strain HOA73 might have potential for postharvest fruit pathogen control. However, the purified chitinase PeChi68 did not fully inhibit the germination of spores of the two tomato pathogens tested. It showed partial inhibition for spores of B. cinerea. Because P. elgii strain HOA73 also produces protocatechagic acid, which is a germination inhibitor for B. cinerea (Nguyen et al., 2015), we believe that an array of compounds could contribute to the biocontrol activity of this bacterium. Therefore, the genome of P. elgii strain HOA73, like that of P. elgii B69, might possess genes encoding several chitinolytic enzymes as well as polyketide synthase, nonribosomal peptide synthase, and lantibiotic synthetic cluster involved in the biosynthesis of antimicrobial compounds (Ding et al., 2011).

In summary, our results on a purified chitinase PeChi68 from P. elgii strain HOA73 suggest that it might play a vital role in the biocontrol of P. elgii strain HOA73 for certain fungal pathogens. Results on its thermal and pH stability suggest that it would be active under a range of environmental conditions. Future studies are needed to determine whether this chitinase may work synergistically with other products from P. elgii strain HOA73 to achieve greater efficacy as biocontrol agents.
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