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ORIGINAL PAPER

# Purification and characterization of chitinases from *Paecilomyces variotii* DG-3 parasitizing on *Meloidogyne incognita* eggs

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**Abstract** Two extracellular chitinases were purified from *Paecilomyces variotii* DG-3, a chitinase producer and a nematode egg-parasitic fungus, to homogeneity by DEAE Sephadex A-50 and Sephadex G-100 chromatography. The purified enzymes were a monomer with an apparent molecular mass of 32 kDa (Chi32) and 46 kDa (Chi46), respectively, and showed chitinase activity bands with 0.01% glycol chitin as a substrate after SDS-PAGE. The first 20 and 15 N-terminal amino acid sequences of Chi32 and Chi46 were determined to be Asp-Pro-Typ-Gln-Thr-Asn-Val-Val-Tyr-Thr-Gly-Gln-Asp-Phe-Val-Ser-Pro-Asp-Leu-Phe and Asp-Ala-X-X-Tyr-Arg-Ser-Val-Ala-Tyr-Phe-Val-Asn-Trp-Ala, respectively. Optimal temperature and pH of the Chi32 and Chi46 were found to be both 60°C, and 2.5 and 3.0, respectively. Chi32 was almost inhibited by metal ions $Ag^{+}$ and $Hg^{2+}$ while Chi46 by $Hg^{2+}$ and $Pb^{2+}$ at a 10 mM concentration but both enzymes were enhanced by 1 mM concentration of $Co^{2+}$. On analyzing the hydrolyzates of chitin oligomers [$(GlcNAc)_n$, $n = 2–6$)], it was considered that Chi32 degraded chitin oligomers as an exo-type chitinase while Chi46 as an endo-type chitinase.

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## Introduction

Fungi produce various isoforms of chitinase with different biophysical functions including lysis of the cell walls (separation of cells after division, hyphal autolysis), nutritional requirements, morphogenetic formation (sporulation, spore germination, and hyphal growth), antagonistic, and parasitic actions against other organisms [31, 33]. On the basis of their amino acid sequences and three-dimensional structures, fungal chitinases belong to glycoside hydrolase family 18, class III with a catalytic $(\alpha/\beta)_8$ barrel domain [6, 7, 9]. Furthermore, according to the mode of their action on chitooligommers and chitin polymers, they can be classified as endochitinases, chitobiosidases, and $\beta$-N-acetyl-glucosaminidases [5].

Fungal chitinases are produced by mycoparasitic, nematophagous, and entomopathogenic fungi [2, 30, 35]. Chitinases from mycoparasitic fungi have been suggested to play a role in host infection [22, 23]. Chitinases from the genus *Trichoderma* have been characterized and cloned, and their expression was monitored when parasitizing other fungi [24, 34, 43]. Chitinases from entomopathogenic fungi can be partially responsible for host penetration [2, 14]. Chitinases from nematode egg-parasitic fungi are believed to be important and absolutely essential for degrading chitinous eggshells [10, 16, 26]. Chitin provides architectural reinforcement of biological structures of insect exoskeletons [19], fungal cell walls [1], and nematode eggshells [3], so that chitinases play a key role in the mechanism of parasitism into host cells [5].

For mycoparasitic fungi, research on purification of chitinases and their role in parasitism is mainly to be focused on the enzymes system of *Trichoderma* spp. [42, 43]. Some extracellular chitinases from entomopathogenic fungi have been considered to be important for pathogenicity. The 33, 43.5, 45, and 60 kDa chitinases from *Metarhizium anisopliae*, and 45.0 kDa endochitinase from *Beauveria bassiana* have been purified and characterized [14, 19]. Compared with extracellular chitinases of mycoparasitic and entomopathogenic fungi, only a few from nematophagous fungi including 52 kDa chitinase from *Paecilomyces lilacinus* [15] and 43 kDa from *Pochonia chlamydosporium* (syn. *Verticillium chlamydosporium*) and *P. suchlasporium* (syn. *V. suchlasporium*) [35] have been purified and studied for elucidation of their role in parasitism of the fungi.

In this paper, two chitinases (Chi32 and Chi46) from *Paecilomyces variotii* DG-3, a chitinase producer and *Meloidogyne incognita* egg-parasite in vitro (27, 28), have been purified and characterized from the culture supernatant of DG-3.

## Materials and methods

### Fungal isolate and identification

Nematode egg-parasitic fungus, *P. variotii* DG-3 was isolated from the soil samples obtained on cucumber crop field in Daegu, Korea. The fungal isolate was maintained at 4°C on potato dextrose agar plates and subcultured every 2 months. For identification of DG-3 isolate, the fungal morphologies: conidia, conidiophores, and colonies were observed [40]. Next, the phylogenetic analysis of the fungus was performed following the method of White et al. [41] and described by Nguyen et al. [29]. Briefly, the genomic DNA was extracted by the G-spinTM Genomic DNA Extraction Kit (Intron Inc., Suwon, Korea). The polymerase chain reaction (PCR) was performed in a GeneAmp Thermal Cycler (Model 2400, Perkin–Elmer, Norwalk, CT, USA). The partial 18S rRNA fragment of the DG-3 isolate was amplified with the universal primers (ITS1-F, 5′-CTTGGTCATTTAGAGGAAGT-3′, and ITS4-R, 5′-CCTCCGCTTATTGATATGC-3′). The nucleotide sequence of 18S rRNA gene of the DG-3 isolate was determined using an Applied Biosystem DNA automated sequencer (ABI373, PE Applied Biosystem, Foster City, CA, USA) then aligned with reference sequences obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The closest similarity of the nucleotide sequences from NCBI database compared with these of DG-3 isolate was chosen by the tree-making software program.

### Chitinase production

The fungus DG-3 was cultured in 0.5% swollen chitin broth medium to produce chitinases. Culture medium containing 5 g swollen chitin [25], 4 g $(NH_4)_2SO_4$, 0.25 g $CaCl_2$, 0.25 g $MgSO_4$ $7H_2O$, 1 g peptone, 2 ml trace element (0.5 g $FeSO_4$ $7H_2O$, 0.15 g $MnSO_4$ $H_2O$, 0.15 g $ZnSO_4$ $7H_2O$, 0.2 g $CoCl_2$ in 100 ml $H_2O$) per liter was inoculated with ten pieces of 5 mm fungal block and incubated in an orbital shaking incubator at 150 rpm, 25°C for 12 days. The culture broth was centrifuged at 6,000 rpm at 4°C for 30 min and then filtered through a No. 2 Whatman filter paper. Concentration of reducing sugar from culture supernatant was measured by Schales' method [11] and amount of protein was estimated by Bradford' assay [4] with bovine serum albumin as a standard. For chitinase activity, the reaction mixture containing 900 µl of 1% swollen chitin (DD 8%) in 50 mM sodium acetate buffer (pH 5.0) and 100 µl of crude enzyme was incubated at 37°C for 1 h, and then stopped by addition of 200 µl of 1 N NaOH. Reaction mixture was centrifuged at 10,000 rpm for 5 min and then the supernatant was used to measure reducing sugar groups. One unit of enzyme activity was defined as the amount of enzyme which produced 1 µM of reducing sugar per hour [17].

### Purification of chitinases from *P. variotii* DG-3 culture filtrate

DG-3 culture filtrate (800 ml) was dialyzed in cellulose membrane (D9420-100FT, Sigma-Aldrich Co. USA) against distilled water for 24 h, concentrated by polyethylene glycol 6000 and then freeze-dried [32]. The protein preparation was dissolved in 20 ml of sterile distilled water and applied to DEAE-Sephadex A-50 column (2.5 × 50 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The enzyme was eluted stepwise with 0.0–0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.5) at a flow rate 1 ml $min^{-1}$. The fractions (5 ml) were collected and assayed for protein content and chitinase activity. F-1 and F-3 chitinase fractions were then pooled and applied to Sephadex G-100 column (1.5 × 50 cm). Protein was eluted with 20 mM Tris–HCl buffer (pH 7.5) at a flow rate of 0.5 ml $min^{-1}$ [13]. Chitinase fractions were collected, pooled and concentrated by centrifugation (6,000 rpm, 20 min) with Amicon 10,000 membrane system (Millipore, USA) for further studies such as SDS-PAGE, amino acid sequencing and enzyme characterization.

### Electrophoresis and activity staining of chitinases

Sodium dodecyl sulfate-polyacrylamine gel electrophoresis (SDS-PAGE) was performed on a 12.5% (w/v) self-poured

polyacrylamine gel containing 0.01% glycol chitin, following the method of Laemmli [18]. Proteins were visualized by staining with 0.12% Coomassie Brilliant Blue R-250. For chitinase activity staining after SDS-PAGE, the opposite gel with the same condition of SDS-PAGE was incubated for 2 h at 37°C in sodium acetate buffer (pH 5.0) containing 1% Triton X-100 (v/v) and 1% skim milk to remove the SDS [13]. The reaction of substrate and chitinases in gel was carried out at 37°C for 24 h in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and then, the gel was stained with 0.01% Fluorescent Brightener 28 in 500 mM Tris–HCl buffer (pH 8.9) for 7 min and sequentially washed with water for at least four times. Activity enzyme bands were visualized as dark bands by a UV transilluminator.

### N-Terminal amino-acid sequencing of chitinases and database searching

Chi32 and Chi46 were electrophoretically transferred from SDS-PAGE to 0.45 μm polyvinylidene-difluoride (PVDF) membrane (Immobilion-P; Millipore, Bedford, CA) using Bio-Rab semidry transfer cell (Bio-Rad, Richmond, CA). Electrobloting was carried out with transfer buffer (50 mM tris-glycine, 20% methanol) at constant current (18 volt) for 1 h. The protein bands were excised from gel and N-terminal amino-acid sequences were analyzed by Prosice model 491 automation N-terminal sequencer (Applied Biosystems) [27]. The data was analyzed using BLAST program of NCBI database.

### Characterization of the enzyme

To determine optimal temperature for chitinase activity, the Chi32 or Chi46 was incubated with 1% swollen chitin in 50 mM citrate buffer (pH 3.0, the optimal pH) at various temperatures from 30 to 80°C for 1 h. To determine optimal pH, the mixture of enzymes and 1% swollen chitin in 50 mM citrate buffer (pH 2–6) was incubated for 1 h. For determining substrate specificity, 100 μl of enzymes was incubated with 900 μl of 0.5% swollen chitin from different sources, ethylene glycol chitin (052-07123, Wako Chemicals, Osaka, Japan) or 0.5% soluble chitosan [12]. The reaction mixtures were incubated at 37°C for 1 h, and enzyme activity was determined as described previously [17]. For specifying the effect of the cations on chitinase activity, a reaction mixture of 900 μl 1% swollen chitin (pH 3.0) plus various cations with a final concentration of 1 mM or 10 mM and 100 μl of Chi32 or Chi46 was incubated at 37°C for 1 h, and the remain enzyme activity was determined as described previously [17].

### Enzymatic hydrolysis of chitooligosaccharides by Chi32 and Chi46

Chitooligomers $[(GlcNAc)_n\ n = 2–6]$ at concentration of 100 μg $ml^{-1}$ in 50 mM citrate buffer (pH 3) were used for analysis of cleavage pattern of Chi32 and Chi46. The reaction mixtures (500 μl) containing 50 μl of purified enzyme, Chi32 or Chi46, and 450 μl of substrate solution was incubated at 37°C for 0, 30, 60, 90 and 120 min and terminated by keeping at 0°C. The reaction mixtures were filtered through a 0.2 μm membrane on ice and stored at −20°C until analyzed. Separation of *N*-acetyl-chitooligosaccharides $[(GlcNAc)_n,\ n = 1–6]$ was performed on a column $NH_2$P-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*-acetyl-chitooligosaccharides from column was monitored at 210 nm [17].

## Results

### Identification of *Paecilomyces variotii* DG-3

DG-3 isolate was identified as *Paecilomyces variotii* DG-3 based on morphological characteristics and phylogenetic analysis. On PDA medium, the DG-3 mycelium varies from yellow color on the first 5 days to tan color with age, and conidia formed in chains with subglobose shape. Based on morphological characteristics, DG-3 was assigned to be belonged to *Paecilomyces* genus. Then phylogenetic analysis of DG-3 isolate was placed with other members of the genus *Paecilomyces* from NCBI database. When the nucleotide sequence of 18S rRNA of DG-3 isolate was aligned with those of other fungi of *Paecilomyces* genus by the tree-making software program in NCBI database, DG-3 sequence was closest to that of *P. variotii* (access No. AF 291869) with 92% similarity thus DG-3 isolate was assigned as *P. variotii* DG-3.

### Purification of chitinases from *P. variotii* DG-3 culture filtrate

Culture supernatants of *P. variotii* DG-3 were daily assayed for pH, concentration of reducing sugar, hydrolyzed from glycosidic bonds of chitin polymer in culture medium by DG-3 chitinase, protein content, and chitinase activity (Table 1). DG-3 chitinase showed the highest at day seven, staying at this level for the length of the incubation period. The time course of chitinase activity was simultaneous with the growth of fungus, amount of reducing sugar, and protein content. The pH of the culture supernatant increased during fungal fermentation from 5.0 to 8.3.

**Table 1** pH, reducing sugar, protein and chitinase activity from culture filtrate of *P. variotii* DG-3 over 12 day growth in 0.5% swollen chitin medium

| Incubation time (day) | pH | Reducing sugar (μmol $ml^{-1}$) | Protein content (μg $ml^{-1}$) | Chitinase activity (U $ml^{-1}$) |
|---|---|---|---|---|
| 0 | 5.0 | 0.32 | 0.0 | 0.0 |
| 1 | 5.3 | 0.34 | 0.0 | 0.0 |
| 2 | 5.8 | 0.33 | 3.4 | 0.1 |
| 3 | 6.2 | 0.40 | 11.6 | 0.7 |
| 4 | 6.4 | 0.42 | 18.1 | 1.7 |
| 5 | 7.0 | 0.44 | 23.7 | 2.6 |
| 6 | 7.1 | 0.50 | 25.3 | 3.0 |
| 7 | 7.2 | 0.50 | 28.8 | 3.2 |
| 8 | 8.0 | 0.53 | 31.7 | 3.5 |
| 9 | 8.1 | 0.55 | 31.5 | 3.5 |
| 10 | 8.2 | 0.58 | 32.6 | 3.5 |
| 11 | 8.3 | 0.56 | 35.0 | 3.6 |
| 12 | 8.3 | 0.60 | 34.8 | 3.8 |

Chitinases were purified by anion exchange and gel filtration chromatography. From DEAE-Sephadex chromatography, chitinase activity was detected as three peaks in the step-elution fractions containing of 0.2–0.4 M NaCl concentration and named F-1 (0.2 M NaCl), F-2 (0.2 M NaCl), and F-3 (0.3–0.4 M NaCl) (Fig. 1). Among them, F-1 and F-3 chitinase fractions contained major proteins with different molecular masses and F-2 fraction contained proteins with the same molecular mass of those of F-1 and F-3 fractions shown by SDS-PAGE. Hence, F-2 chitinase fraction was not further purified. F-1 and F-3 chitinase peaks were pooled and then further purified using filtration chromatography. F-1 fraction resulted in a single chitinase activity peak from 22 to 30 fractions (Fig. 2, F-1) and F-3 yielded only one chitinase peak from 11 to 19 fractions (Fig. 2, F-3).

Culture supernatant, flow-through protein fractions and the pooled enzymes of F-1 and F-3 peaks were analyzed by SDS-PAGE and the chitinase activity staining (Fig. 3). Crude enzyme and flow-through fractions yielded several bands and among them 32 kDa and 46 kDa proteins were

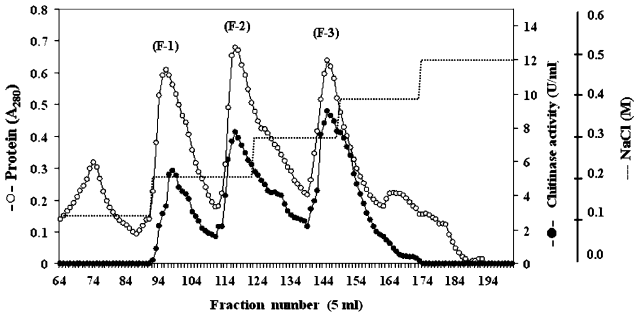


**Fig. 1** DEAE-Sephadex chromatography of *P. variotii* DG-3 culture supernatant. The protein was eluted stepwise with 20 mM Tris-HCl (pH 7.5) containing 0.0–0.5 M sodium chloride

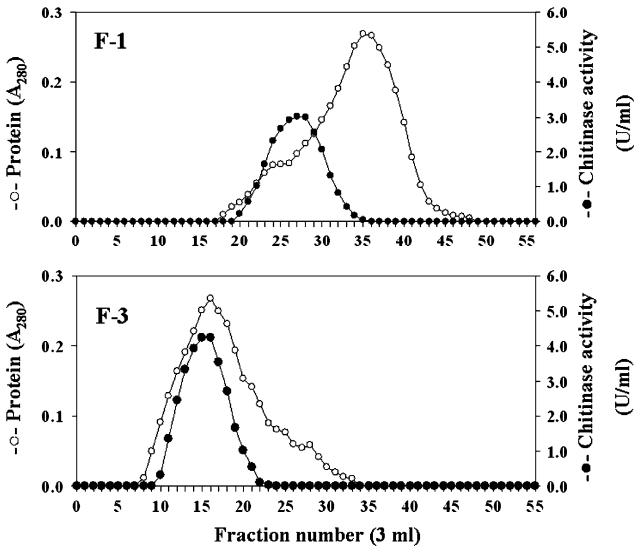


**Fig. 2** Sephadex G-100 chromatography of F-1 chitinase fractions (*F-1*) and *F-3* chitinase fraction (*F-3*) obtained from DEAE-Sephadex chromatography. The protein was eluted with in 20 mM Tris–HCl (pH 7.5)

the major bands showing chitinase activity. After two purification steps of anion exchange and filtration chromatography, F-1 and F-3 chitinases were purified to near homogeneity with MW approximately of 32 and 46 kDa showed by SDS-PAGE and chitinase activity staining, namely, Chi32 and Chi46, respectively (Fig. 3a, b). These protein bands were similar chitinase activity bands exhibited from crude enzyme and flow-through fractions.

## N-Terminal amino acid sequencing

N-Terminal amino-acid sequences of Chi32 and Chi46 revealed that the first 20 and 15 amino acids of these enzymes were Asp-Pro-Typ-Gln-Thr-Asn-Val-Val-Tyr-

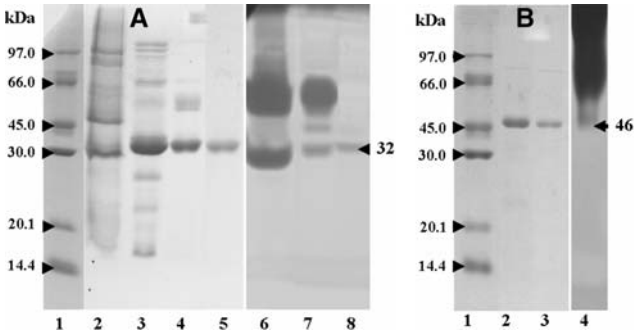


**Fig. 3** SDS-PAGE of Chi32 (**a**) and Chi46 (**b**). Proteins (40 µl each) were loaded in each lane. The gels were stained with Coomassie brilliant blue R-250 for 12% SDS-PAGE and with Fluorescent Brightener 28 for chitinase activity staining as in "Materials and Methods". In panel **a**: *lane 1*, molecular weight marker (Amersham Biosciences); *lane 2*, crude enzyme; *lane 3*, flow- through protein (no bound to DEAE column); *lane* 4, fractions F-1 from DEAE-Sephadex column; *lane 5*, purified Chi32 chitinase from Sephadex G-100 column; *lane 6–8*, activity staining of crude enzyme, fraction F-1, and the purified Chi32. In panel **b**: *lane 1*, molecular weight marker; *lane 2*, fractions F-3 from DEAE-Sephadex column; *lane 3*, purified Chi46 chitinase from Sephadex G-100 column; and *lane 4*, activity staining of the purified Chi46

Thr-Gly-Gln-Asp-Phe-Val-Ser-Pro-Asp-Leu-Phe and Asp-Ala-X-X-Tyr-Arg-Ser-Val-Ala-Tyr-Phe-Val-Asn-Trp-Ala, respectively. The sequence of Chi46 was found to be closest similarity with those of 43 kDa endochitinase from *Hypocrea virens* (accession number AF397021) and *H. jecorina* (accession number DAA05855) [34] with aligned amino acids from 5 to 15 residues in N-terminal sequence while N-terminal amino acids of Chi32 showed low match with other amino acid sequences in NCBI database.

## Characterization of Chi32 and Chi46

The optimal temperature for enzyme activity of Chi32 and Chi46 were found to be both 60°C (Fig. 4a). Above 80°C, the chitinases lost almost all activity. The Chi32 and Chi46 showed the highest activity at a pH of 2.5 to 3.0 (Fig. 4b). The relative chitinase activities at pH 2.0 and 6.0 were only 13.7 and 26.9% for Chi32 and Chi46, respectively.

When the chitinase activity on several chitin-derived substrates was investigated, the highest activity was found on water-soluble chitin (DD 50%) and the lowest activity on crab colloidal chitin (DD 8%) (Table 2). Interestingly, the two chitinases also degraded soluble chitosan (DD 74%). It may occur due to the activity toward the GlcNAc-GlcN and GlcN-GlcNAc linkages retained in chitosan. Table 3 shows the effect of various metal ions at 1 and 10 mM concentration on the activity of Chi32 and Chi46. At the 10 mM concentration, Chi32 was completely inhibited by $Ag^+$ and $Hg^{2+}$ whereas Chi46 was inhibited by $Hg^{2+}$ and $Pb^{2+}$. For both enzymes, activity was enhanced at the 1 mM concentration of $Co^{2+}$.

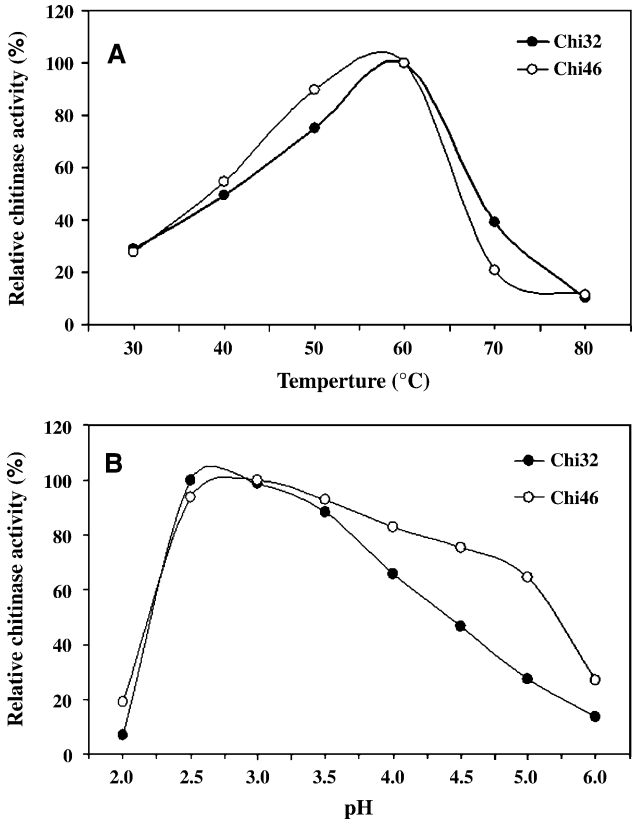


**Fig. 4** Optimal temperature and pH of both Chi32 and Chi46

## Analysis of the hydrolysis of chitin oligosaccharides by Chi32 and Chi46

Chitin dimer $(GlcNAc)_2$, trimer $(GlcNAc)_3$, tetramer $(GlcNAc)_4$, pentamer $(GlcNAc)_5$, and hexamer $(GlcNAc)_6$ were used to analyze the hydrolytic pattern of Chi32 and Chi46 on chitin substrate (Fig. 5). The size of the products formed during the hydrolysis of chitin oligomers by Chi32 and Chi46 were analyzed by high-pressure liquid chromatography (HPLC). It was observed that chitin dimer was not degraded by both Chi32 and Chi46 after up to 120 min of incubation (data not shown). This means that chitin dimer is not the substrate for both enzymes. In reaction of Chi32 for 60 min of incubation, trimer was degraded to $(GlcNAc) + (GlcNAc)_2$, tetramer was hydrolyzed to major production of $(GlcNAc)_2$ and little amount of $GlcNAc + (GlcNAc)_3$ while pentamer was cleaved to $(GlcNAc)_2 + (GlcNAc)_3$ and hexamer was cleaved to major product $(GlcNAc)_2$ and little amount of $GlcNAc + (GlcNAc)_3$ (Fig. 5, left A–D).

Conversely, after incubation of Chi46 with the same substrates, trimer was not practically hydrolyzed while tetramer was cleaved to final products of $(GlcNAc)_2$. Pentamer was cleaved to $(GlcNAc)_2 + (GlcNAc)_3$ with almost equal amount of production. Hexamer was hydrolyzed to

**Table 2** Substrate specificity of the purified Chi32 and Chi46 from *P. variotii* DG-3

| Substrate | Chi32 | | Chi46 | |
|---|---|---|---|---|
| | Chitinase activity (U $ml^{-1}$) | Relative activity (%) | Chitinase activity (U $ml^{-1}$) | Relative activity (%) |
| Water soluble CT (DD 50) | 28.9 | 100.0 | 22.4 | 100.0 |
| Crab swollen CT (DD 8) | 3.7 | 12.7 | 3.8 | 16.9 |
| Shrimp swollen CT | 2.1 | 7.2 | 2.8 | 12.4 |
| Squid swollen CT | 3.5 | 12.1 | 3.5 | 15.4 |
| Crab colloidal CT (DD 8) | 0.3 | 1.1 | 0.2 | 0.9 |
| Ethylene glycol CT | 2.2 | 7.7 | 0.7 | 3.3 |
| Crab powder CT (100 μm, DD 8) | 0.9 | 3.0 | 0.6 | 2.8 |
| Soluble CTS (DD 74) | 8.5 | 29.4 | 10.5 | 46.7 |

*CT* chitin, *CTS* chitosan, *DD* degree of deacetylation

**Table 3** Effect of metal ions on the activity of the purified Chi32 and Chi46 from *P. variotii* DG-3

| Metal ion | Chi32 | | | | Chi46 | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 mM | | 10 mM | | 1 mM | | 10 mM | |
| | Chitinase activity (U $ml^{-1}$) | Relative activity (%) | Chitinase activity (U $ml^{-1}$) | Relative activity (%) | Chitinase activity (U $ml^{-1}$) | Relative activity (%) | Chitinase activity (U $ml^{-1}$) | Relative activity (%) |
| No ions | 3.1 | 100.0 | 3.1 | 100.0 | 3.6 | 100.0 | 3.6 | 100.0 |
| $AgNO_3$ | 2.7 | 88.3 | 0.0 | 0.0 | 3.2 | 88.7 | 2.6 | 71.7 |
| $HgCl_2$ | 0.6 | 21.0 | 0.0 | 0.0 | 2.0 | 55.4 | 0.0 | 0.0 |
| $Pb(NO_3)_2$ | 2.5 | 82.1 | 0.9 | 30.5 | 3.5 | 97.4 | 0.0 | 0.0 |
| $MgSO_4$ | 2.9 | 96.1 | 2.7 | 89.1 | 3.6 | 100.0 | 3.3 | 92.8 |
| $MnCl_2$ | 2.8 | 92.2 | 2.3 | 76.0 | 3.6 | 101.0 | 3.1 | 87.1 |
| $CuSO_4$ | 2.6 | 85.2 | 2.0 | 65.4 | 2.7 | 77.0 | 1.8 | 51.1 |
| $FeSO_4$ | 2.9 | 96.1 | 2.6 | 83.5 | 3.6 | 101.4 | 3.4 | 94.7 |
| $ZnCl_2$ | 2.8 | 90.5 | 1.3 | 42.8 | 3.5 | 99.0 | 2.7 | 74.8 |
| $ZnSO_4$ | 2.8 | 91.9 | 1.0 | 33.3 | 3.4 | 96.6 | 1.9 | 52.8 |
| $CoCl_2$ | 4.3 | 141.3 | 2.7 | 88.3 | 4.7 | 130.9 | 2.7 | 74.6 |

$(GlcNAc)_2 + (GlcNAc)_4$ and $(GlcNAc)_3 + (GlcNAc)_3$ (Fig. 5, right A–D).

To further analyze mode of action of both enzymes, a time course of hydrolysis of chitin oligomers was carried out. In reaction of Chi32 with chitin oligomers, GlcNAc and $(GlcNAc)_2$ were the major products from the chitin oligomers for just 30 min of incubation (Fig. 6a–d). It was further shown that hydrolysis product of $(GlcNAc)_3$ fragments from $(GlcNAc)_5$ or $(GlcNAc)_6$ was further hydrolyzed to $(GlcNAc) + (GlcNAc)_2$. In summary, Chi32 is considered as exo-type chitinase.

In case of Chi46, profiles of hydrolysis production were different from those of Chi32 (Fig. 6e–h). Trimer was not degraded over time of incubation but a little amount of GlcNAc and $(GlcNAc)_2$ was shown in production. Hydrolysis product of tetramer was the only dimer for all the time of incubation. Pentamer was almost degraded to main products of $(GlcNAc)_2$ and $(GlcNAc)_3$ and produced $(GlcNAc)_3$ was not further hydrolyzed. In the hydrolysis of hexamer, $(GlcNAc)_3$ and $(GlcNAc)_4$ appeared at early stage and then the produced tetramer was continuously degraded to dimer but trimer fragments were not further degraded over incubation time. From these results, Chi46 is considered as endo-type chitinase.

## Discussion

Fungal chitinases are being considered as a key role in the parasitic mechanism of fungi into nematode eggs [15, 29, 35]. *Paecilomyces variotii* DG-3 was previously screened as a chitinase producer and *Meloidogyne* egg-parasitic agent [28, 29]. For chitinase production, in submerged culture a large number of factors contribute to development of

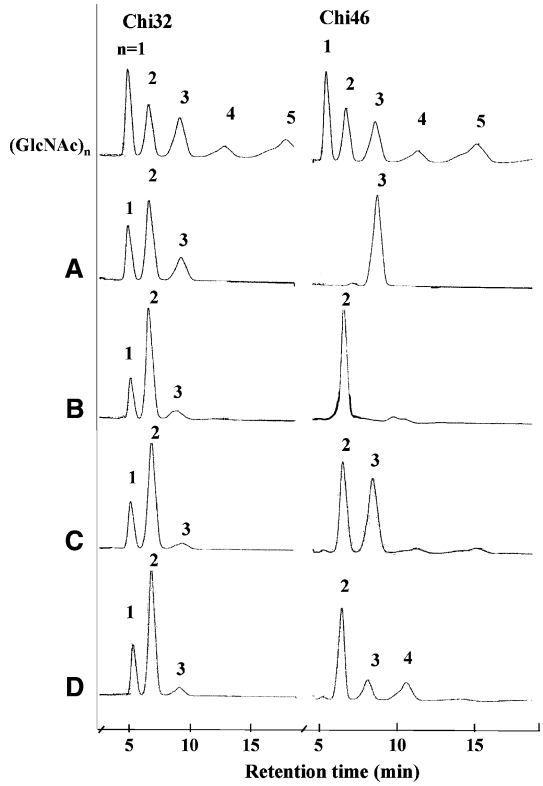


**Fig. 5** Enzymatic hydrolysis products of chitin oligomers by Chi32 (on the *left*) and Chi46 (on the *right*). $(GlcNAc)_n$, Standard chitin oligomer, [$(GlcNAc)_n$ $n$ = 1–6]; *A* hydrolysis products from trimer $(GlcNAc)_3$, *B* tetramer $(GlcNAc)_4$, *C* pentamer $(GlcNAc)_5$, and *D* hexamer $(GlcNAc)_6$. Mixture of 450 µL of substrate (100 µg $ml^{-1}$) in 50 mM citrate buffer (pH 3) and 50 µL of enzymes were incubated at 37°C for 60 min. The products (3 µL) were separated by HPLC

any particular morphological forms of fungi and the chitinase production. In our studies, the fermentation medium was supplemented with 0.5% swollen chitin as nutritional trigger for *P. variotii* to produce chitinases. Chitinase activity of DG-3 in the culture medium increased during the first 6 days and then kept plateau until 12 days. Tikhonov et al. [35] reported that chitinase activity of *P. chlamydosporium* and *P. suchlasporium* on semiliquid colloidal chitin medium increased over the course of 2 weeks. In this work, a 12 day fungal culture was used for enzyme purification.

The two chitinases, Chi32 and Chi46, were purified by DEAE-Sephadex and Sephadex G-100 chromatography which resulted in a relatively low yield when compared to previous reports [20]. Both enzymes showed chitinase activity on a gel containing 0.01% glycol chitin as substrate after SDS-PAGE. For detecting in-gel chitinase activity after SDS-PAGE, glycol chitin, carboxymethyl-chitin-remazol brilliant violet 5R (CM-chitin-RBV), or 4-methyl-umbelliferyl-($N$-acetyl-$\beta$-D-glucosamine)$_{1-3}$ (4-MU-GlcNAc)$_{1-3}$ are used as substrates [6, 37–39]. Glycol chitin, a soluble modified form of chitin, has been commonly used as a substrate for detecting almost endochitinases [37] and (4-MU-GlcNAc)$_{1-3}$ for detecting exochitinases including chitobiosidase and $\beta$-N-acetyl-glucosaminidase [37]. The 36 kDa chitobiosidase from *Bacillus cereus* was detected in-gel using glycol chitin as a substrate [39]. Chi32 and Chi46 clearly displayed enzyme activity bands in the gel containing 0.01% glycol chitin (Fig. 3).

Both enzymes Chi32 and Chi46 showed their highest enzyme activity at 60°C. This falls within the established temperature range for fungal chitinases reported by Sahai and Manocha [33]. Temperature is a major factor regulating on the folding/unfolding behavior of protein and thus effects on binding affinity between substrate and enzyme [21, 36]. The pH value influences the proton-donating or -accepting groups in the catalytic site of enzymes. Both Chi32 and Chi46 had their optimum under acidic conditions at a pH value of 2.5–3.0, respectively. This finding was confirmed by using two pH buffers, sodium acetate buffer (pH 3–5) and citrate buffer (pH 2–6), respectively. This is the first report for the fungal chitinase having optimal pH at such low acidic values. Previous work reported optimal pH values for fungal chitinases between 4 and 8 [20, 33]. Metal ions play a role as activators or inhibitors. Chi32 and Chi46 were activated by 41.3 and 30.9%, respectively, at 1 mM concentration of $Co^{2+}$. It was considered the $Co^{2+}$ could modulate the conformation of active center. Heavy metal ion ($Ag^{+}$, $Hg^{2+}$, and $Pb^{2+}$) inhibited Chi32 or Chi46 activity at 1 mM concentration.

The N-terminal amino acid sequence of Chi46 contained two unidentified amino acid XX (residue No. 3 and 4) but exhibited complete homology with those of chitinase gene products of *Hypocrea jecorina* (accession number DAA05855) and *H. virens* (accession number AF397021) from amino acid residue No. 5. The sequence of Chi32 was also compared against the NCBI database. It showed little homology with other known sequences, probably suggesting that Chi32 is a newly reported chitinase.

On analyzing the hydrolytic pattern of chitin polymers by chitinases, endochitinases randomly cleave chitin to produce chitin oligomers, such as chitotetraose, chitotriose, and chitobiose. Chitobiosidases, one of exochitinases, split out the chitin chain either from the reducing or nonreducing end to produce chitobiose, while $N$-acetyl-$\beta$-D-glucosaminidases hydrolyse chitobiose and higher analogues from terminal nonreducing end to produce GlcNAc [5, 20]. In a traditional classification of endo- and exo-chitinases, chitin oligomers could be used as a substrate and their hydrolyzed products could be examined [8]. In the case of Chi46, chitin tetramer was degraded to $(GlcNAc)_2$, pentamer to $(GlcNAc)_2 + (GlcNAc)_3$ and hexamer to $(GlcNAc)_2 + (GlcNAc)_3 + (GlcNAc)_4$, while Chi32 degraded chitin

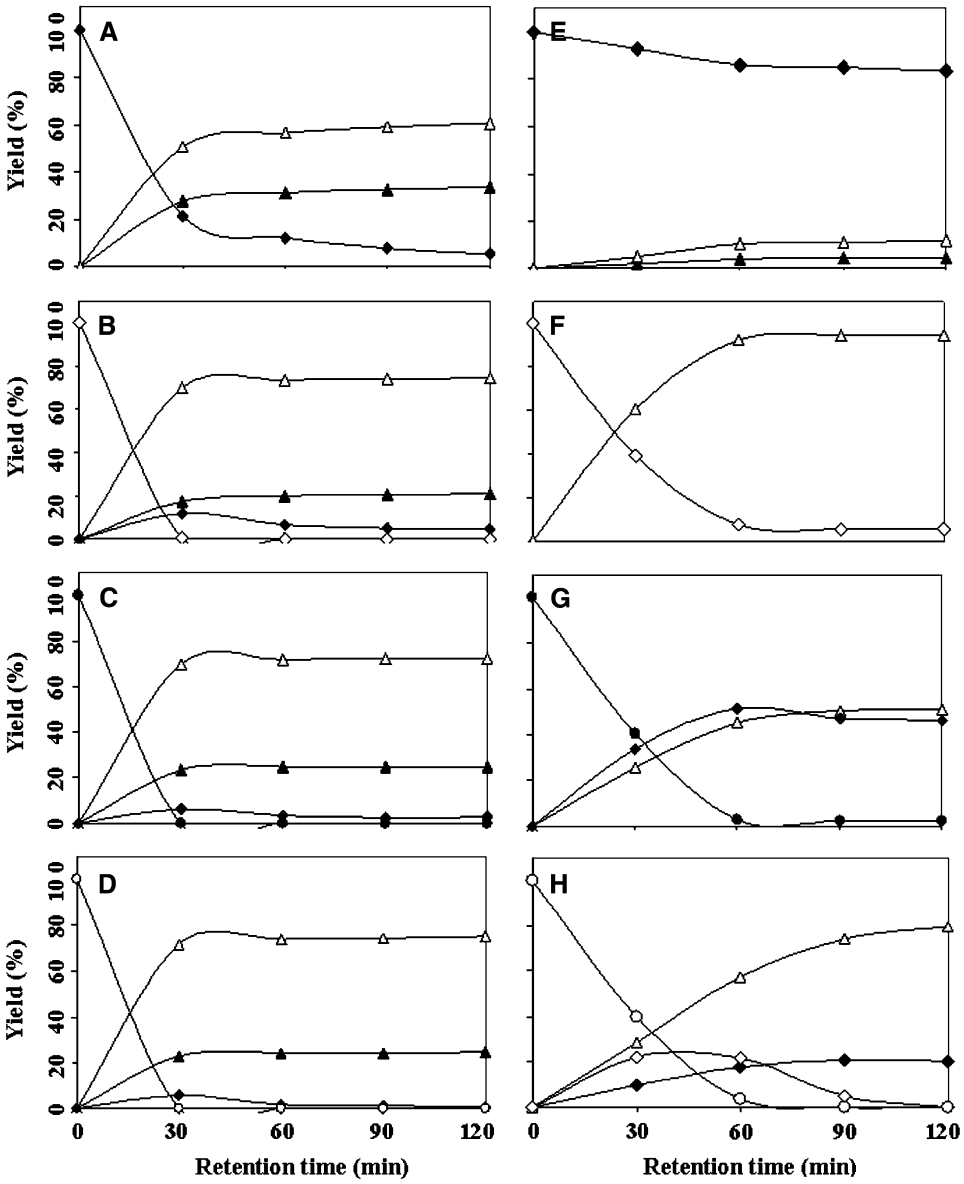


**Fig. 6** Time course of product formation by Chi32 (on the *left*) and Chi46 (on the *right*) from chitin trimer (**a**, **e**); tetramer (**b**, **f**), pentamer (**c**, **g**), and hexamer (**d**, **h**). Mixture of 450 μL of substrate (500 μg $ml^{-1}$) in 50 mM citrate buffer (pH 3) and 50 μL of enzymes were incubated at 37°C for 0, 30, 60, 90 and 120 min. The products (3 μL) were separated by HPLC. *Filled triangle* GlcNAc, *open triangle* $(GlcNAc)_2$, *fillrd diamond* $(GlcNAc)_3$, *open diamond* $(GlcNAc)_4$, *filled circle* $(GlcNAc)_5$, *open circle* $(GlcNAc)_6$

oligomers $(GlcNAc)_{3–6}$ to GlcNAc and $(GlcNAc)_2$ and chitin dimer was not degraded by Chi32. Consequently, Chi46 and Ch32 were characterized as endochitinase and exochitinase, respectively.

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