

## Purification and Characterization of Chitinase from *Paenibacillus illinoisensis* KJA-424

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Received: April 20, 2004

Accepted: July 2, 2004

**Abstract** A chitinase was purified from the culture supernatant of *Paenibacillus illinoisensis* KJA-424 by protein precipitation, DEAE-Sephadex anion-exchange chromatography, and Sephadex G-150 gel filtration. The molecular weight of the purified chitinase was 54 kDa on SDS-PAGE and activity staining. Optimal pH and temperature were pH 5.0 and 60°C, the presence of 10 mM Ag<sup>+</sup> and Hg<sup>2+</sup> inhibited the activity by 92.1% and 97.7%, and the  $K_m$  and  $V_{max}$  values were 1.12 mg chitin ml<sup>-1</sup> and 1.48 μmol GlcNAc min<sup>-1</sup>, respectively. The enzyme hydrolyzed tetramer to dimer, pentamer to dimer and trimer, and hexamer to dimer, trimer and tetramer, indicating an endo-splitting mechanism. The chitinase had no hydrolytic activity toward dimer and trimer. The chitinase inhibited the mycelial growth of *Rhizoctonia solani*, suggesting an antifungal property.

**Key words:** *Paenibacillus illinoisensis*, *Rhizoctonia solani*, antifungal chitinase

Chitinase has the ability to degrade chitin, a major component of most fungal cell walls. The chitinolytic enzymes are classified as endochitinase, exochitinase (EC 3.2.1.14), *N*-acetyl-β-D-glucosaminidase, and chitobiase (EC.3.2.1.30). Endochitinases exhibit nonspecific random cleavage of the chitin polymer and chitin oligomers to release smaller, soluble *N*-acetyl-β-D-glucosamine (GlcNAc) oligomers of variable size [12, 17, 24, 31]. The second type proposed are chitin β-1,4-chitobiosidases (chitobiosidase), which are now newly classified as exochitinases. These enzymes cleave chitin chains at the nonreducing end, releasing diacetylchitobiose molecules. The third group, β-*N*-acetylhexosaminidases (EC 3.2.1.52), progressively break down chitin and

chitoooligosaccharides from the nonreducing end of the molecule, releasing a monomer, GlcNAc.

Chitinolytic enzymes, together with cellulase or β-1,3-glucanase, are the enzymes most frequently considered to be critical in biocontrol [4]. Chitinases are lytic enzymes capable of inhibiting fungal development by degrading the pathogen's cell wall, which is composed mostly of chitin and glucan [1, 23]. Chitinolytic bacterial strains suppress *Fusarium* wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* in nonsterile, soilless potting medium [25]. Native fluorescent pseudomonads have been screened *in vitro* for their antagonistic activity against the phytopathogenic fungi such as *Pythium ultimum* and *Rhizoctonia solani* [2]. Chitin has been used to control fungal diseases and root-parasitic nematodes, possibly due to increase of chitinase-producing microorganisms in the environment [26].

The objectives of this study were to purify a chitinase from *Paenibacillus illinoisensis* KJA-424, which was identified in our previous studies as a potential antagonist against *Rhizoctonia solani* [14], and to characterize its properties from a biocontrol viewpoint for pathogens.

## MATERIALS AND METHODS

### Chemicals

Ammonium sulfate, bovine serum albumin, carboxymethyl-chitin, glycol chitin, glycol chitosan, *N*-acetyl-β-D-glucosamine, standard molecular weight markers, and Calcofluor white M2R were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). DEAE-Sephadex A-50 and Sephadex G-150 resin were purchased from Pharmacia LKB (Uppsala, Sweden), chromatography column and protein assay dye-reagent concentrates were from Bio-Rad (Richmond, CA, U.S.A.), and chitin oligosaccharides, (GlcNAc)<sub>n</sub> (n=2-6), were from Wako Chemicals (Osaka, Japan).

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### Organism and Culture

*Paenibacillus illinoisensis* KJA-424 was isolated from a coast soil in Korea [13], and a pure culture was maintained in 25% glycerol at  $-80^{\circ}\text{C}$ . To produce chitinase, the seed culture was inoculated into a 2.0-l Erlenmeyer flask, containing 1 l of basal medium (0.2%  $\text{Na}_2\text{HPO}_4$ , 0.1%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{NH}_4\text{Cl}$ , 0.05%  $\text{NaCl}$ , 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 0.05% yeast extract, pH 7.0) supplemented with 0.2% colloidal chitin, and the culture was incubated at  $30^{\circ}\text{C}$  for 4 days in a shaking incubator (180 rpm). The culture broth was centrifuged at  $8,000 \times g$  and  $4^{\circ}\text{C}$  for 30 min. The supernatant was used for the purification of the enzyme.

### Enzyme Purification

The culture supernatant was first treated with 80% ammonium sulfate. After overnight, the precipitate formed was centrifuged at 12,000 rpm for 30 min. The precipitate was dissolved in a small volume of sterile distilled water and dialyzed against distilled water overnight at  $4^{\circ}\text{C}$ . The dialysate (3 ml) was applied to DEAE-Sephadex A-50 column (1.5 $\times$ 50 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.2), and the column was washed with 2 bed volumes of equilibrium buffer. The enzyme was eluted stepwise with 0.1–0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.2). The fractions with chitinase activity were pooled and concentrated with polyethylene glycol (# 6,000) at  $4^{\circ}\text{C}$ . The concentrated enzyme (2.0 ml) was then applied to Sephadex G-150 column (1.5 $\times$ 50 cm), which was eluted with 20 mM Tris-HCl (pH 8.2) buffer at a flow rate of 0.5 ml/min. The fractions of chitinase activity were pooled and concentrated with polyethylene glycol at  $4^{\circ}\text{C}$ .

### Determination of Chitinase Activity

Chitinase activity was determined by measuring the amount of reducing end group, GlcNAc (*N*-acetyl- $\beta$ -D-glucosamine) degraded from colloidal chitin, as described by Lingappa and Lockwood [20]. The assay mixture consisted of 0.2 ml of enzyme suspension, 0.5 ml of 1.0% colloidal chitin, and 0.5 ml of 100 mM sodium acetate buffer (pH 5.5). After incubation at  $37^{\circ}\text{C}$  for 1 h, 200  $\mu\text{l}$  of 1 N NaOH were added to stop the reaction. After brief centrifugation, 750  $\mu\text{l}$  of supernatant were mixed with 1 ml of Schales' reagent (0.5 M sodium carbonate+1.5 mM potassium ferricyanide), and the mixture was heated in boiling water for 15 min. Absorbance at 420 nm was measured using a spectrophotometer (Shimadzu, UV-1601, Japan). The amount of reducing sugar was calculated from a standard curve obtained from known concentrations of GlcNAc (0–100  $\mu\text{g}$ ). One unit of chitinase activity was defined as the amount of enzyme to liberate 1  $\mu\text{mol}$  of *N*-acetylglucosamine per h. Protein content was determined by the method described by Bradford [3] with bovine serum albumin as a reference protein. Protein was also monitored by the absorbance at 280 nm during chromatographic separation. Colloidal chitin was

prepared from commercial chitin by the method of Godoy *et al.* [6].

### SDS-PAGE and Activity Staining

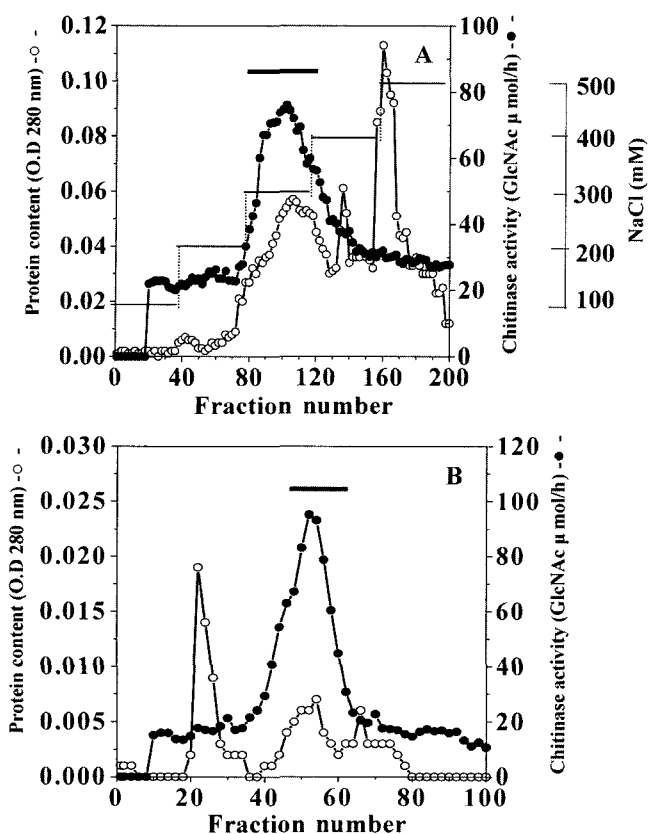
SDS-PAGE was performed, according to the method described by Laemmli [15], with the Bio-Rad Mini Protein II apparatus (50 $\times$ 100 $\times$ 1.5 mm), using 12.5% (w/v) polyacrylamide separating gels and 5% (w/v) polyacrylamide stacking gel. Proteins were stained with 0.12% Coomassie brilliant blue R-250. Chitinase activity staining was done, according to the method described by Trudel and Asselin [28]. After SDS-PAGE, the gel was incubated for 2 h at  $37^{\circ}\text{C}$  with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.0), containing 1% (v/v) Triton X-100 and 1% skim milk. Then, the gel was again incubated at  $37^{\circ}\text{C}$  for 24 h with shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100. The gel was immersed into 500 mM Tris-HCl (pH 8.9) solution containing 0.01% Calcofluor white M2R (Sigma F3397). The zones lysed by enzymes were visualized and photographed on a UV transilluminator.

### Characterizations of Purified Chitinase

For pH effect, the enzyme with colloidal chitin as a substrate was incubated in 50 mM citrate buffer at pH 3, 100 mM sodium acetate buffer at pH 4–5, 200 mM phosphate buffer at pH 6–8, and 200 mM sodium carbonate buffer at pH 9–10 for 1 h. For temperature effect, the enzyme was incubated with colloidal chitin in sodium acetate buffer (pH 5.0) at different temperatures ranging from 20 to  $70^{\circ}\text{C}$  for 1 h. For substrate specificity, activity of the enzyme on several chitin-derived substrates and nonchitin substrate was determined. The substrates tested were colloidal chitin, carboxymethyl cellulose, carboxymethyl chitin, glycol chitin, and glycol chitosan in 0.1 M sodium acetate buffer (pH 5.0) for  $37^{\circ}\text{C}$  for 1 h. For metal ionic effect, the enzyme was incubated with colloidal chitin plus 10 mM metal ion in 50 mM sodium acetate buffer (pH 5.0) at  $37^{\circ}\text{C}$  for 1 h. For kinetic constants, 100  $\mu\text{l}$  of enzyme preparation (10 mU) were incubated with various concentrations of colloidal chitin, between 0.25 and 2.5 mg/ml at  $37^{\circ}\text{C}$  for 1 h. Michaelis-Menten constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) were determined by Lineweaver-Burk transformation. For analysis of enzyme reaction product, the reaction mixture (500  $\mu\text{l}$ ) contained the purified chitinase and substrate (100  $\mu\text{g}$  for chitin oligomers and 500  $\mu\text{g}$  for swollen chitin) in 100 mM sodium acetate buffer (pH 5.5), and was incubated at  $37^{\circ}\text{C}$  for 1 h. The reaction products were spotted on silica gel thin-layer plates. After developing in *n*-propanol:water:ammonia solution (70:30:1, v/v), *N*-acetyl- $\beta$ -D-glucosamine sugars were visualized by the silver nitrate-sodium hydroxide reaction [11].

### Mycelial Growth Inhibition

The inhibition of mycelial growth by the purified chitinolytic enzymes was tested. *Rhizoctonia solani* was grown in



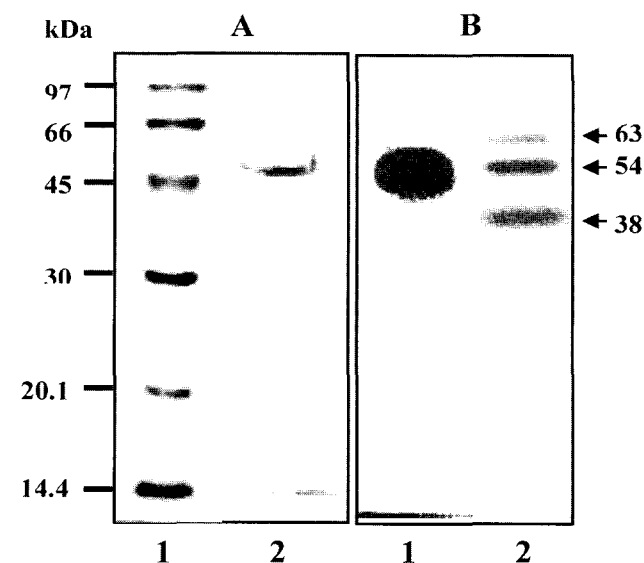
**Fig. 1.** Chromatograms of DEAE-Sephadex column (A) and Sephadex G-150 column (B) chromatographies. Fractions indicated by the dashed line were pooled and concentrated.

potato dextrose agar (PDA), and one piece of *R. solani* disk (1 cm diameter) was then placed in the center of the plates, and then the purified enzyme (2  $\mu$ g of protein) was spotted on the edge of the plates. The plates were incubated at 27°C for 3 days, and the distances between the edges of the enzyme and fungal mycelium were observed.

## RESULTS AND DISCUSSION

### Cell Growth and Chitinase Production

The cell growth of *P. illinoisensis* KJA-424 in the 0.2% colloidal chitin containing broth medium increased rapidly to the highest level within 2 days, and the same level was maintained thereafter. Chitinase activity was parallel with



**Fig. 2.** SDS-polyacrylamide gel electrophoresis of proteins purified from *P. illinoisensis* KJA-424.

Panel A. Stained with Coomassie brilliant blue R-250. 1, Molecular mass marker; 2, fractions (1  $\mu$ g protein) partially purified from Sephadex G-150 column. Panel B. Activity-stained with Calcofluor white M2R. 1, Partially purified fraction (1  $\mu$ g protein) from Sephadex G-150; 2, crude protein (10  $\mu$ g) from culture broth.

bacterial growth for the first 3 days, and then decreased to about two third of the maximal level. Maximal specific activity of extracellular chitinase was 2.1 units  $\text{mg protein}^{-1}$ , when the culture reached a cell density of 0.8 (OD 600 nm) on the second day of culture. The maximum activity was achieved when the initial pH of the medium was adjusted to 7.0 (data not shown).

### Purification of Chitinolytic Enzymes

DEAE-Sephadex chromatography after 80% ammonium sulfate precipitation resulted in three protein peaks (Fig. 1A). Among them, the fractions by 300 mM NaCl contained most of the activity. The specific activity of chitinase at this step was 14.45 units  $\text{mg}^{-1}$  protein with a purification factor of 6.95. Sephadex G-150 gel filtration resulted in a single chitinase activity (Fig. 1B). The specific activity of the purified chitinase was 17.5 units  $\text{mg}^{-1}$  protein with a purification factor of 8.41 (Table 1).

### Detection of Chitinase Activity

After gel filtration, the protein pooled from the fractions of 45 to 60 was subjected to SDS-PAGE: A single band of

**Table 1.** Purification of chitinase from *Paenibacillus illinoisensis* KJA-424.

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Purification factor	Recovery (%)
Crude enzyme	8.00	16.63	2.08	1.00	100.0
80% $(\text{NH}_4)_2\text{SO}_4$	2.08	13.61	6.54	3.14	81.9
DEAE-Sephadex	0.29	4.19	14.45	6.95	25.2
Sephadex G-150	0.12	2.10	17.50	8.41	12.6

54 kDa was stained with Coomassie Blue R-250 (Fig. 2A). When the gel was stained with Calcofluor white M2R after SDS-PAGE containing glycol chitin (0.01%), only one band of chitinase activity was detected at the position of 54 kDa (Fig. 2B). On the other hand, the culture medium contained three bands (38, 54, and 63 kDa) with chitinase activity (Fig. 2C). Chitinases purified from various microorganisms have been reported to have varying sizes of molecular mass, such as 50 kDa for *Bacillus* sp. LJ-25 [17], 45 kDa for *Bacillus circulans* No. 4.1 [31], 54 kDa for *Pseudomonas* YHS-A2 [16], and 66.5 kDa for *Bacillus* sp. 7079 [8]. Also, Park *et al.* [22] demonstrated that chitinolytic enzyme from *Chromobacterium violaceum* strains was composed of four isoforms of 54, 52, 50, and 37 kDa.

### Characterization of Chitinase Enzyme

The optimal pH for chitinolytic activity of KJA-424 was found to be 5.0 (Fig. 3A) and the optimal temperature was 60°C (Fig. 3B). Similar optimal pH was obtained; that is, pH 4.0 of *T. harzianum* strain P1 [9], pH 4.5 of *T. harzianum* strain 39.1 [29], and between pH 3.0 and 6.0

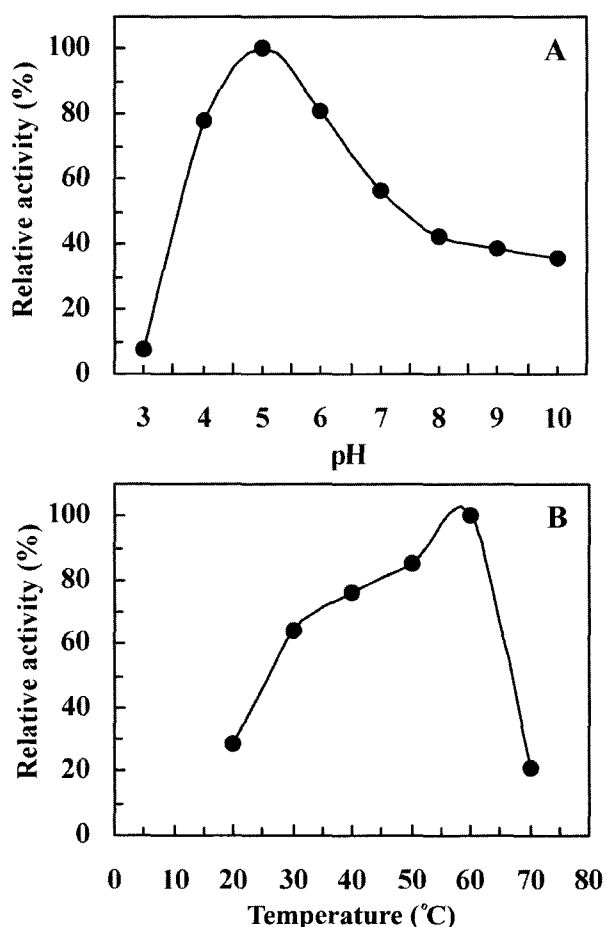


Fig. 3. The pH (A) and temperature (B) profiles of chitinase purified from *P. illinoisensis* KJA-424.

Table 2. Substrate specificity of the chitinase purified from *P. illinoisensis* KJA-424.

Substrate	Enzyme activity ( $\mu\text{mol min}^{-1}$ )	Relative activity (%)
Colloidal chitin	1.118	100.0
CM-chitin	0.299	26.7
Glycol chitin	0.412	36.8
Glycol chitosan	0.007	0.6
Chitosan	0.051	4.6
CM-cellulose	0.290	25.9

of *Pseudomonas aeruginosa* K-187 [30]. The chitinase from *P. aeruginosa* K-187 was stable up to 100°C, and about 90% of its activity remained even after 30 min at high temperature.

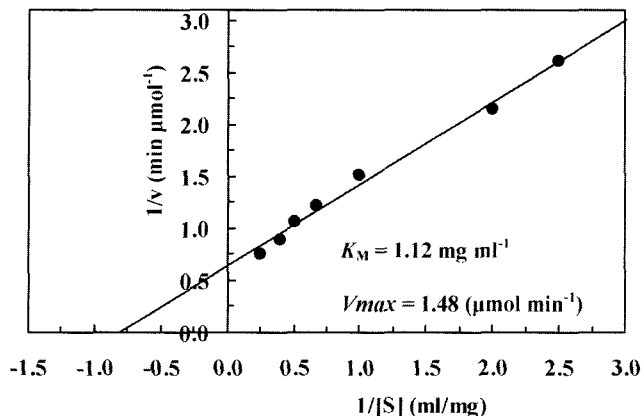
The activity of the chitinase on several chitin-derived substrates was investigated (Table 2). It showed the highest activity on the colloidal chitin, being 3.7-fold and 2.7-fold higher than those on CM-chitin and glycol-chitin, respectively. Colloidal chitin was more susceptible to the chitinase of *Bacillus circulans* No. 4.1 than CM-chitin [31]. However, some studies demonstrated that CM-chitin was the most susceptible to *Trichoderma harzianum* T198 chitinase [5], whereas glycol-chitin was the most susceptible to *Bacillus circulans* No.4.1 chitinase [31] and *Bacillus licheniformis* TP-1 [27].

The activity of the purified enzyme in the presence of various metallic ions was assessed (Table 3). The metal ions such as  $\text{Ag}^+$  and  $\text{Hg}^{2+}$  inhibited the activity by 92.1% and 97.7%, respectively. Yabuki *et al.* [32] suggested that sulfhydryl groups are vital for chitinolytic enzymes from *Aeromonas hydrophila* subsp. *anaerogenes* A52.

From the Lineweaver-Burk plot, the kinetic parameters,  $K_m$  and  $V_{max}$ , of chitinolytic enzyme were calculated to be 1.12 mg chitin  $\text{ml}^{-1}$  and 1.48  $\mu\text{mol GlcNAc min}^{-1}$ , respectively, for the hydrolysis of colloidal chitin (Fig. 4). The above  $K_m$  value is comparable to the values of 1.28 and 3.68 mg chitin  $\text{ml}^{-1}$  for the chitinases from *Aeromonas salmonicida* YA7-625 [18] and *Serratia* sp. 3095 [19], respectively. The  $K_m$  and  $V_{max}$  values for *T. harzianum* chitinase using chitotriose as a substrate were found to be 850  $\mu\text{M}$  and  $1.16 \times 10^{-1} \mu\text{mol ml}^{-1} \text{h}^{-1}$ , respectively [5].

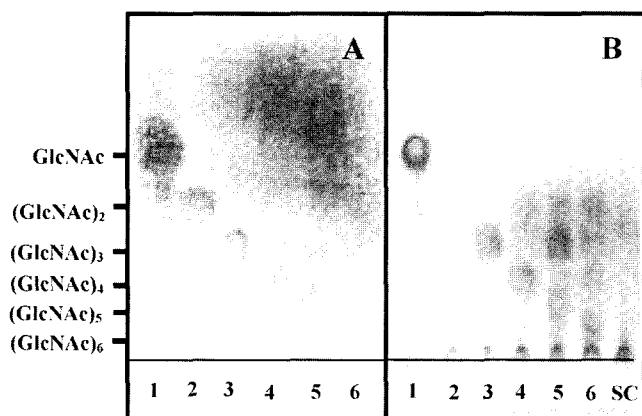
Table 3. Effect of heavy metal ions on the chitinase from *Paenibacillus illinoisensis* KJA-424.

Metal ions	Relative activity (%)
None	100.0
$\text{Pb}^{2+}$	82.4
$\text{Mg}^{2+}$	93.3
$\text{Ag}^{2+}$	7.9
$\text{Hg}^{2+}$	2.3



**Fig. 4.** Lineweaver-Burk plot of *P. illinoisensis* KJA-424 activity. Purified enzyme was incubated for 1 h at 37°C with a range of concentrations of 0.5% colloidal chitin in 50 mM sodium acetate buffer (pH 5.0).

Using chitin oligomers as substrates, the enzymatic mechanism was investigated. As seen in Fig. 5, tetramer was cleaved to dimer, and pentamer to dimer and trimer. Hexamer was degraded to dimer, trimer, and tetramer in an endo-splitting manner. Chitin dimer and trimer were not degraded by the enzyme even after prolonged reactions (Fig. 5). The minimum size of substrates, that the KJA-424 chitinase could hydrolyse was tetramer. Swollen chitin was degraded to chito oligomers, (GlcNAc)<sub>2,6</sub>, and the main products were dimer and trimer (Fig. 5). These results indicate the endo-splitting mechanism of the KJA-424 chitinase. Monomer was never detected in the enzyme reaction.



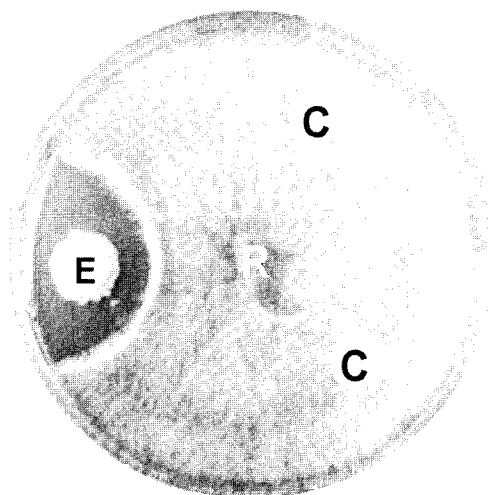
**Fig. 5.** TLC chromatogram of enzyme reaction products from chitin oligosaccharides.

The purified enzyme was added to each oligomer (5 μg/μl) dissolved in 100 mM sodium acetate buffer (pH 5.5). After incubation at 30°C for 24 h, samples were chromatographed as described in Materials and Methods. (A) Lanes 1–6: Authentic chitin oligomers (GlcNAc)<sub>1–6</sub>. (B) Lanes 1: N-acetyl glucosamine (Standard); 2: products from dimer; 3: products from trimer; 4: products from tetramer; 5: products from pentamer; 6: products from hexamer; SC: products from swollen chitin.

Similar to our result, acidic chitinase from *Citrus sinensis* L. exhibited endochitinase activities, as oligosaccharide (GlcNAc)<sub>4–6</sub> was digested into smaller ones; that is, tetramer to (GlcNAc)<sub>2</sub>, pentamer to (GlcNAc)<sub>2–3</sub>, and hexamer to (GlcNAc)<sub>2</sub> or (GlcNAc)<sub>3</sub> [21]. Chitinase from *Aeromonas* sp. No. 16 hydrolyzed colloidal chitin predominantly to (GlcNAc)<sub>2</sub>, and trace amounts of (GlcNAc)<sub>1</sub> and (GlcNAc)<sub>3</sub> [11]. The thermostable chitinase from *Bacillus licheniformis* KFB-C14 could hydrolyze chitin oligosaccharides into (GlcNAc)<sub>2</sub> [10].

### Mycelial Growth Inhibition

Antifungal assays *in vitro* were carried out on PDA plates treated with 20 μl (2 μg proteins) of chitinolytic enzymes purified on Sephadex G-150 column. As shown in Fig. 6, the enzyme completely inhibited the mycelial growth of *R. solani* after 3 days of inoculation, possibly due to the endo-chitinolytic activity of the KJA-424. Lee *et al.* [16] reported that chitinase purified from *Pseudomonas* sp. YHS-A2 inhibited the growth of some phytopathogenic fungi, such as *Fusarium oxysporum*, *Botrytis cineria*, and *Mucor rouxii*, and Han *et al.* [7] reported that chitinase of *Bacillus amyloliquefaciens* 7079 exhibited strong antifungal activity. Several studies have shown that efficient parasitic biocontrol agents excrete extracellular lytic enzymes that are responsible for their antagonistic abilities [25]. Chitinase, β-1,3-glucanase, and laminarinase, by acting alone or synergistically, have been shown to inhibit the growth of pathogenic fungi by degradation or lysis of fungal cell walls [23]. Thus, it is highly likely that mycelial growth inhibition of *R. solani* might mainly be due to the endo-chitinolytic activity of *P. illinoisensis* KJA-424.



**Fig. 6.** Antifungal activity of the chitinase from *P. illinoisensis* KJA-424.

C, Sterilized water (control); E, Purified enzyme obtained from Sephadex G-150; R, *Rhizoctonia solani*.

## Acknowledgment

This study was supported by National Research Laboratory (NRL) program (to Dr. R.-D. Park) from the Ministry of Science & Technology (MOST), Korea.

## REFERENCES

- Arlorio, M., A. Ludwig, T. Boller, and P. Bonfante. 1992. Inhibition of fungal growth by plant chitinase and 1,3-glucanase. *Protoplasma* **171**: 34–43.
- Bagnasco, P., L. De La Fuente, G. Gualtieri, F. Noya, and A. Arias. 1998. Fluorescent *Pseudomonas* spp. as biocontrol agents against forage legume root pathogenic fungi. *Soil Biol. Biochem.* **30**: 1317–1322.
- Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.* **72**: 248–254.
- Chet, I. 1987. Trichoderma - Application, mode of action, and potentials biological agent of soil-borne plant pathogenic fungi, pp. 137–160. In I. Chet (ed.), *Innovative Approaches to Plant Disease Control*. John Wiley & Sons. New York, U.S.A.
- Deane, E. E., J. M. Whipps, J. M. Lynch, and J. F. Peberdy. 1998. The purification and characterization of a *Trichoderma harzianum* exochitinase. *Biochem. Biophys. Acta* **1383**: 101–110.
- Godoy, G., R. Rodríguez-Kábana, and G. Morgan-Jones. 1982. Parasitism of eggs of *Heterodera glycines* and *Meloidogyne arenaria* by fungi isolated from cysts of *H. glycines*. *Nematropica* **12**: 111–119.
- Han, O. K., E. T. Lee, and S. D. Kim. 2001. Chitinase of multifunctional antagonistic bacterium *Bacillus amyloliquefaciens* 7079 against phytopathogenic fungi. *Kor. J. Appl. Microbiol. Biotechnol.* **29**: 142–148.
- Han, O. K., E. T. Lee, Y. S. Lee, and S. D. Kim. 2003. Purification of chitinase from an antagonist bacterium *Bacillus* sp. 7079 and pro-inflammatory cytokine gene expression by PCTC. *J. Microbiol. Biotechnol.* **13**: 77–84.
- Harman, G. E., C. K. Hayes, M. Lorito, R. M. Broadway, A. Di Pietro, C. Peterbauer, and A. Tronsmo. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. *Phytopathology* **83**: 313–318.
- Hong, B. S., H. G. Yoon, D. H. Shin, and H. Y. Cho. 1996. Purification and characterization of thermostable chitinase from *Bacillus licheniformis* KFB-C14. *Kor. J. Appl. Microbiol. Biotechnol.* **24**: 567–573.
- Huang, J. H., C. J. Chen, and Y. C. Su. 1996. Production of chitinolytic enzymes from a novel species of *Aeromonas*. *J. Ind. Microbiol.* **17**: 89–95.
- Jo, Y. Y., K. J. Jo, Y. L. Jin, W. J. Jung, J. H. Kuk, K. Y. Kim, T. H. Kim, and R. D. Park. 2003. Characterization of endochitinase-producing *Bacillus cereus* P16. *J. Microbiol. Biotechnol.* **13**: 960–968.
- Jung, W. J., S. J. Jung, K. N. An, Y. L. Jin, R. D. Park, K. Y. Kim, B. K. Shon, and T. H. Kim. 2002. Effect of chitinase-producing *Paenibacillus illinoisensis* KJA-424 on egg hatching of root-knot nematode (*Meloidogyne incognita*). *J. Microbiol. Biotechnol.* **12**: 865–871.
- Jung, W. J., K. N. An, Y. L. Jin, R. D. Park, K. T. Lim, K. Y. Kim, and T. H. Kim. 2003. Biological control of damping-off caused by *Rhizoctonia solani* using chitinase-producing *Paenibacillus illinoisensis* KJA-424. *Soil Biol. Biochem.* **35**: 1261–1264.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–683.
- Lee, H. S., H. J. Lee, S. W. Choi, S. Her, and D. H. Oh. 1997. Purification and characterization of antifungal chitinase from *Pseudomonas* sp. YHS-A2. *J. Microbiol. Biotechnol.* **7**: 107–113.
- Lee, J. S., D. S. Joo, S. Y. Cho, J. H. Ha, and E. H. Lee. 2000. Purification and characterization of extracellular chitinase produced by marine bacterium, *Bacillus* sp. LJ-25. *J. Microbiol. Biotechnol.* **10**: 307–311.
- Lee, K. P., C. N. Kim, J. H. Yu, and D. H. Oh. 1990. The production and purification of chitinase from *Aeromonas salmonicida* YA7-625. *Kor. J. Appl. Microbiol. Biotechnol.* **18**: 599–606.
- Lee, E. T. and S. D. Kim. 1999. Purification and characterization of antifungal chitinase from indigenous antagonistic microorganism *Serratia* sp. 3095. *Agric. Chem. Biotechnol.* **42**: 7–11.
- Lingappa, Y. and J. L. Lockwood. 1962. Chitin media for selective isolation and culture of actinomycetes. *Phytopathology* **52**: 317–323.
- Möder, W., A. Bunk, A. Albrecht, H. Doostdar, R. P. Niedz, R. E. McDonald, R. T. Mayer, and W. F. Osswald. 1999. Characterization of acidic chitinase from culture medium of sweet orange callus tissue. *J. Plant Physiol.* **154**: 296–301.
- Park, S. K., H. Y. Lee, and J. W. Huh. 1995. Production and some properties of chitinolytic enzymes by antagonistic bacteria. *Kor. J. Plant Pathol.* **11**: 258–264.
- Punja, Z. K. and Y. Y. Zhang. 1993. Plant chitinase and their role in resistance to fungal disease. *J. Nematol.* **25**: 526–540.
- Sakai, K., A. Yokota, H. Kurokawa, M. Wakayama, and M. Moriguchi. 1998. Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain, MH-1, isolated from chitin-containing compost. *Appl. Environ. Microbiol.* **64**: 3397–3402.
- Singh, P. P., Y. C. Shin, C. S. Park, and Y. R. Chung. 1999. Biological control of fusarium wilt of cucumber by chitinolytic bacteria. *Phytopathology* **89**: 92–99.
- Spiegel, Y., I. Chet, and E. Cohn. 1987. Use of chitin for controlling plant-parasitic nematodes II. Mode of action. *Plant Soil* **98**: 337–345.
- Tantimavanich, S., S. Pantuwatana, A. Bhumiratana, and W. Pannbangred. 1998. Multiple chitinase enzymes from a single gene of *Bacillus licheniformis* TP-1. *J. Ferment. Bioeng.* **85**: 259–265.

28. Trudel, J. and A. Asselin. 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* **178**: 362–366.
29. Ulhoa, C. J. and J. F. Peberdy. 1992. Purification and some property of the excellular chitinase produced by *Trichoderma harzianium*. *Enzyme Microb. Technol.* **14**: 236–240.
30. Wang, S. L., T. C. Yieh, and I. L. Shih. 1999. Production of antifungal compounds by *Pseudomonas aeruginosa* K-187 using shrimp and crab shell powder as a carbon source. *Enzyme Microb. Technol.* **25**: 142–148.
31. Wiwat, C., S. Patcharaporn, and B. Amarat. 1999. Purification and characterization of chitinase from *Bacillus circulans* No. 4.1. *Curr. Microbiol.* **39**: 134–140.
32. Yabuki, M., K. Mizushina, T. Amatatsu, A. Ando, T. Fujii, M. Shimada, and M. Yamashida. 1986. Purification and characterization of chitinase and chitobiose produced by *Aeromonas hydrophila* subsp. *Anaerogenes* A52. *J. Gen. Appl. Microbiol.* **32**: 25–38.