Purification and Charaterization of Antifungal Chitinase from Indigenous Antagonistic Microorganism *Serratia* sp. 3095

Eun-Tag Lee and Sang-Dal Kim*

Department of Applied Microbiology, College of Natural Resources, Yeungnam University, Kyongsan 712-749, Korea.

Received January 25, 1999

An extracellular chitinase of the selected strong antifungal microorganism, *Serratia* sp. 3095, was purified by salting out, affinity adsorption, Sepadex G-100 gel fitration, Sepadex G-75 gel fitration and DEAE Sepadex A-50 chromatography. The molecular weight of the purified chitinase was estimated to be 62,000 dalton by SDS-PAGE. Optimal pH and temperature of the chitinase were pH 7.5 and 45, respectively. The enzyme retained more than 80% of the activity between pH 5.5 and pH 10.5, and below 50°C but was unstable above 60°C, below pH 5.0. The activity of the chitinase was inhibited about 60% by Sn^{2+}, 40% by Hg^{2+} and Ag^{+}, 70% by AHA, 40% by iodoacetate, 35% by thiourea and *p*-CMB, but stabilized by SDS. K_m value of the purified chitinase was 3.68 mg/ml for colloidal chitin. The chitinase from *Serratia* sp. 3095 showed antifungal activity to *Fusariurm solani*.

Key words : *indigenous antagonistic microorganism,* Serratia, *chitinase, antifungal.*

Fusarium solani which is a causal phytopathogenic fungi of plant root-rot has chitin-abundant hyphal wall. Chitin, which is one of the most abundant biomass in nature next to cellulose and is constructed with β-1,4-linked polymer of *N*-acetylglucosamin, has been found in crab shell, insect crust and fungal cell wall as skeletion materials.[1-6] In soil ecosystem, many chitinase producing antagonistic microorganisms are able to inhibite fusarial phytopathogenic fungi such as *F. solani* or *F. oxysporum* with their extracellular chitinase.[7-10] The chitinase has an important antifungal biological controling activity for the reduction of fungal plant disease besides antifungal antibiotics and antagonistic sidrophores.[11-13]

We were able to select and identify a novel indigenous antifungal bacterium *Serratia* sp. 3095 which can produce extracellular chitinase with high antifungal activity as a powerful biological control agent, as reported previously.[14] We purified the antifungal chitinase from antifungal bacterium *Serratia* sp. 3095 and investigated its enzymatic characteristics. Also the antifungal ability of the chitinase was investigated with fungi-bacterium dual culture and *in vitro* pairing culture.

*Corresponding author
Phone: 82-53-810-2395; Fax: 82-53-811-4319
E-mail: sdkim@yeungnam.ac.kr

Abbreviations : AHA, acetohydroxamic acid; CMC, sodium carboxymethyl cellulose; CMM, chitin-minimal medium; *p*-CMB, *p*-chloromercuribenzoic acid; 2,4-DNP, 2,4-dinitrophenol; DNS, 3,5-dinitrosalicylic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEM, transmission electron microscopy.

Materials and Methods

Bacterial strain and medium. Indigenous antagonistic microorganism (*Serratia* sp. 3095), isolated and selected from Kyungju A-Hwa region, was cultivated in King's B medium at 30°C for 2 days and stored at 4°C. The strain was identified by API® test, Identification system of Biolog Co., various biochemical test and TEM.[14] The colloidal chitin was preparated by the modified Jeuniaux method.[15] In order to estimate the antifungal activity of the strain, *Serratia* sp. 3095 and *F. solani* were cultivated with a dual culture in liquid medium and a pairing culture in plate media.

Chitinase production. For the production of chitinase, *Serratia* sp. 3095 was cultivated in the CMM [0.1% pepton, 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.01% $MgSO_47H_2O$, 0.15% colloidal chitin] for 72 hrs at 30°C and 180 rpm. After centrifugation for 20 min at 10,000×*g*, culture supernatant were used for chitinase assay.

Enzyme and protein assay. Chitinase activity was measured by DNS method[16] using colloidal chitin as a substrate. The reaction mixture [1 ml 1/15 M phosphate buffer (pH 7.5), 0.5 ml 0.5% colloidal chitin and 0.1 ml enzyme solution] was incubated in a shaking-waterbath at 45°C for 90 min.

One unit of enzyme activity was defined as the amount of enzyme which produce 1 μM glucose equivalent for 1 hr from colloidal chitin under the above condition. Protein concentration was determined by the Lowry methods[17] with bovine serum albumin as a standard.

Production of crude enzyme. The chitinase producing

strain, *Serratia* sp. 3095 was cultivated in CMM for 3 days at 30°C and 180 rpm on a shaking incubator. After cultivation, the cells were removed by centrifugation at 10,000×*g* for 20 min. Ammonium sulfate was added to the supernatant as powder to obtain 75% saturation. After standing for 12 hrs at 4°C, the precipitate was collected by centrifugation (10,000×*g*, 20 min) and dissolved with a minimum volume of the buffer (1/15 M phosphate buffer, pH 7.5). This solution was dialyzed again in the same buffer to obtain.

Chitinase purification by affinity adsorption. To the crude enzyme solution, the same volume of 1% (w/w) colloidal chitin solution was added and gently vortexed for 1 hr at 4°C on cold chamber. This mixture was centrifuged at 10,000×*g* for 20 min and the supernatant was discarded. The precipitate was washed using 1/15 M phosphate buffer (pH 7.5) by centrifugating at 10,000×*g* for 20 min and the supernatant was discarded. After adding an equal volume of the 1/15 M phosphate buffer (pH 7.5) to the precipitate and then vortexing it, the colloidal chitin in the mixture by incubation at 45°C for 3 hrs. It was centrifuged at 10,000×*g* for 20 min and then the supernatant was centrifuged for 50 min at 3,000×*g* using Centriprep® 10 filter.

Sephadex G-100 chromatography. The concentrated sample was applied to a Sephadex G-100 column (2.5×80 cm) equilibrated with 1/15 M phosphate buffer (pH 7.5) and eluted with same buffer. Active fractions were pooled and concentrated.

Sephadex G-75 chromatography. The concentrated active fractions was applied to a Sephadex G-75 column (1.5×100 cm) equilibrated with 1/15 M phosphate buffer (pH 7.5) and eluted with same buffer.

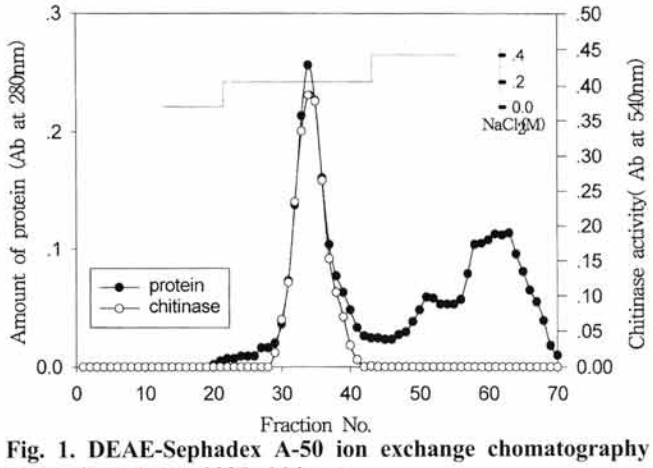

Fig. 1. DEAE-Sephadex A-50 ion exchange chomatography of the *Serratia* sp. 3095 chitinase.

Ion exchange chromatography. The concentrated active fraction was applied to DEAE-Sephadex A-50 column (1.5× 50 cm) and eluted by stepwise elution of NaCl (0, 0.2, 0.4 N). Activity fractions were pooled and lyophilized.

Electrophoresis. SDS-PAGE was done according to Laemmli methods, using 10% gel. After electrophoresis, gel was stained by 0.2% Coomassie brilliant blue R-250. Destaining was with a 7% acetic acid and 5% methanol mixture.

Antifungal activity of *Serratia* sp. 3095. In order to confirm of antifungal activity of the *Serratia* sp. 3095 against *F. solani*, two strains were cultured to dual culture and *in vitro* pairing culture methods. And breaking of *F. solani* mycellium was observed by TEM.

Results and Discussion

Chitinase purification. Enzyme from *Serratia* sp. 3095 was purified by 75% ammonium sulfate precipitation, affinity absorption, gel filtration (Sephadex G-100, G-75) and ion exchange chromatography (DEAE-Sephadex A-50). Chitinase was purified about 14.2-fold with an overall yield of 19.9% (Table 1). The purified chitinase showed single band on disc-electrophoresis (data not shown) and SDS-PAGE. The molecular weight was estimated to be about 62,000 dalton (Fig. 2).

The molecular weight of the chitinase was similar to other chitinise size which was 33,000 of *Ewingella americana*,[18] 54,900 of *Penicillium oxalicum*,[19] 52,000 of *Serratia marcescens*[20] and 50,000 of *Aspergillus fumigatus*.[21] But the 200.000 of *Aeromonase salmonicida* was very different to that of *Serratia* sp. 3095.

Substrate specificity. The activities of the purified chitinase on chitin, glycolchitin, colloidal chitin, chitosan, glycolchitosan and other polysaccharides were presented in Table 2. These results indicated that the chitinase show high substrate specificity of colloidal chitin.

Effects of pH and temperature. The optimal temperature and pH for *Serratia* sp. 3095 chitinase were examined. The enzyme was most active at 45°C and pH 7.5 under standard assay condition (Fig. 3A, B). These results indicate that chitinase produced by *Serratia* sp. 3095 is similar to *A. salmonicida*,[23] *A. fumigatus* chitinase[21] but different to *P. oxalicum* chitinase (35°C, pH 5.0).[19] In order

Table 1. Summary of the purification of the *Serratia* sp. 3095 chitinase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Recovery (%)
Culture filtrate	1,600.1	6,386.0	4.0	-	100.0
Salting out and dialysis	752.9	5,186.4	6.9	1.7	81.2
Affinity adsorption	366.6	3,638.2	9.9	2.5	57.0
Sephadex G-100 gel filtration	84.7	1,768.2	20.9	5.2	27.7
Sephadex G-75 gel filtration	64.2	1,534.2	24.0	6.0	24.0
DEAE-Sephadex A-50 chromatography	22.4	1,272.8	56.8	14.2	19.9

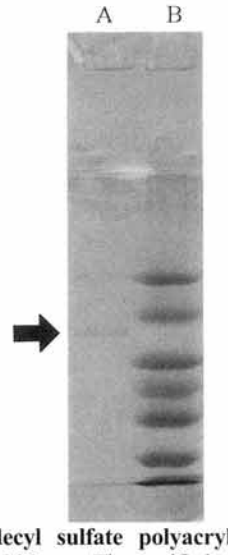

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified chitinase. The purified chitinase was electrophoresised on a 10% polyacrylamide slab gel with 0.1% SDS. Lane A, Purified chitinase. Lane B, Molecular weight markers (97.4 kDa, Phosphorylase B; 66.2 kDa, Bovine Serum Albumin; 55.2 kDa, Glutamate Dehydrogenase; 42.7 kDa, Ovalbumin; 40.0 kDa, Aldolase; 31.0 kDa, Carbonic anhydrase; 21.5 kDa, Soybean Trypsin Inhibitor).

Table 2. Substrate specificity of the *Serratia* sp. 3095 chitinase.

Substrate	Relative activity (%)
α-Cellulose	0
Lichenan	0
D-Cellobiose	0
CMC	1.4
Starch	0.6
Chitin	4.7
Chitosan	0.2
Colloidal chitin	100

to determine the effect of pH on the stability of the chitinase, enzyme was incubated in pH buffer 2~12 for 12 hrs at 4°C. The remaining activity was determined. The results were shown in Fig. 4A. The enzyme was stable at pH 5.5~10.5. To determine the heat stability of the chitinase, the enzyme solution in 1/15 M phosphate buffer (pH 7.5) was incubated at various temperatures for 10 to 60 min. After addition of colloidal chitin to the enzyme solution, the assay was performed. The results were shown in Fig. 4B. The remaining activities after treatment of the enzyme at 70°C and 60°C for 20 min were 10% and 20%, respectively. About 80% of initial activity was remained even after incubation for 60 min at 50°C. But the enzyme activity was relatively unstable over 60°C.

Effect of metal ions and chemical reagents. To determine the effect of metal ions and chemical reagents, each reagent was added to the enzyme solution at a final concentration of 1 mM. The chitinase assay was done after preincubation of the enzyme mixture for 12 hrs at 4°C.

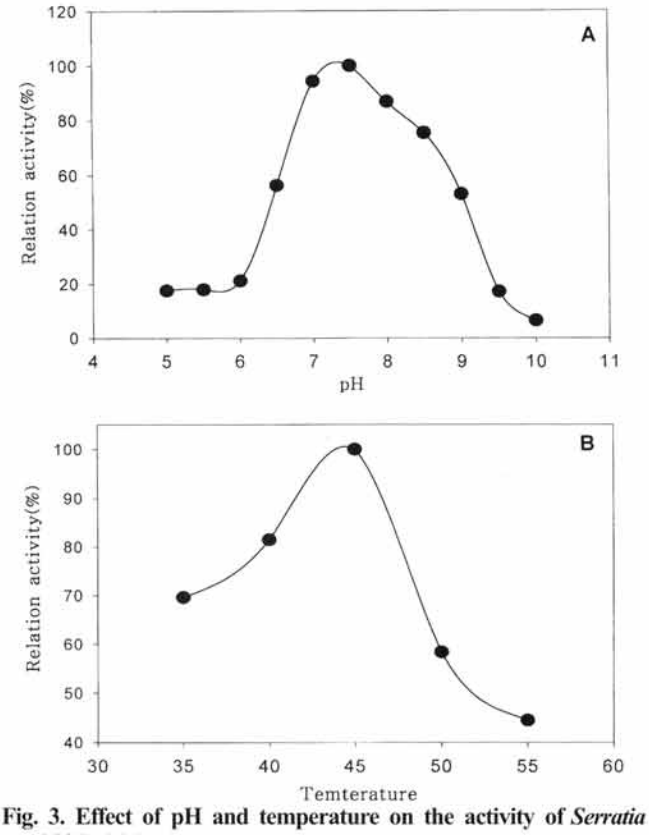

Fig. 3. Effect of pH and temperature on the activity of *Serratia* sp. 3095 chitinase.

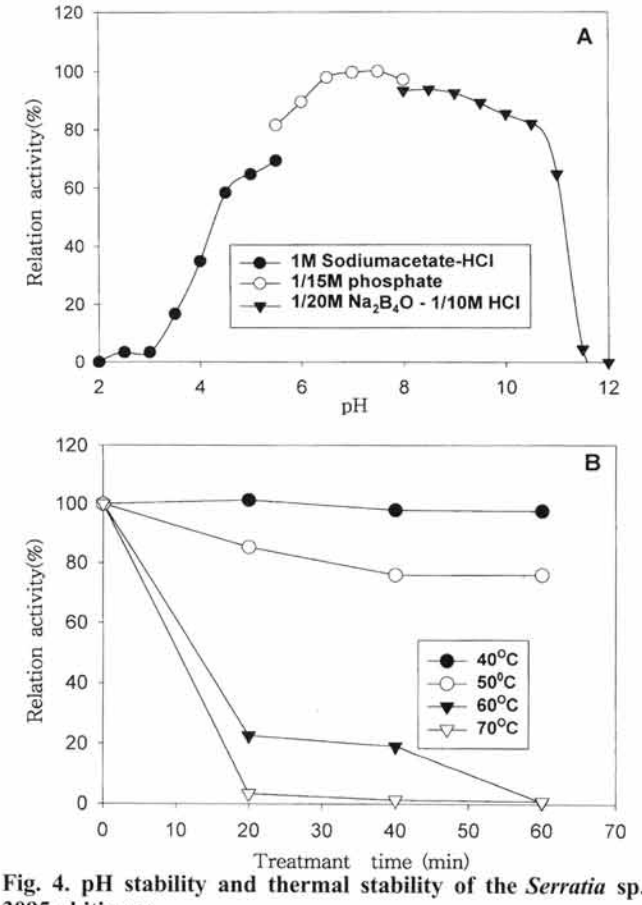

Fig. 4. pH stability and thermal stability of the *Serratia* sp. 3095 chitinase.

Table 3. Effect of various enzyme inhibitors and metal ions on the *Serratia* sp. 3095 chitinase.

Chemical/metal ions	Residual activity (%)
None	100.0
SDS	92.0
p-CMB	68.2
Iodoacetic acid	60.2
EDTA	70.8
CDTA	72.7
L-cystein	78.0
Hydroxyurea	65.9
AHB	31.8
Sodium azide	75.0
$MgSO_4 \cdot 7H_2O$	65.5
$CuSO_4 \cdot 5H_2O$	69.1
$ZnSO_4 \cdot 7H_2O$	93.6
$FeCl_2 \cdot 6H_2O$	79.2
$HgCl_2$	58.0
$CaCl_2$	87.8
$MnSO_4 \cdot 7H_2O$	92.8
$AgNO_3$	59.2
$Pb(CH_3COO)_2$	63.1
$NiCl_2$	71.2
$BaCl_2$	87.1
$SnCl_2$	40.2

Final concentration of salts 10^{-3}M

Sn^{2+} ions inhibited the enzyme activity about 60% and Hg^{2+}, Ag^{+}, Pb^{2+} ions inhibited about 40%. But Zn^{2+} and Mn^{2+} ions inhibited it about less than 10% (Table 3). The purified chitinase was inhibited of 25~30% by sodium azide, EDTA and CDTA, which suggests that also the chitinase from *Serratia* sp. 3095 is a metaloenzyme. The chitinase was inhibited of 30% and 40% by *p*-CMB and idoacetic acid, which supposed that the chitinase has SH-group at active site. The enzyme was inhibited of 70% by AHB and 30% by thiourea (Table 3).

K_m value. The affinity of the purified chitinase for colloidal chitin was examined with a Lineweaver-Burk plot as shown in Fig. 5. The enzyme exhibited Michaelis-Menten constant (K_m) of 3.68 mg/ml. The chitinase from *Aeromonase salmonicida* YA7-625[22] had K_m value of 1.28 mg/ml.

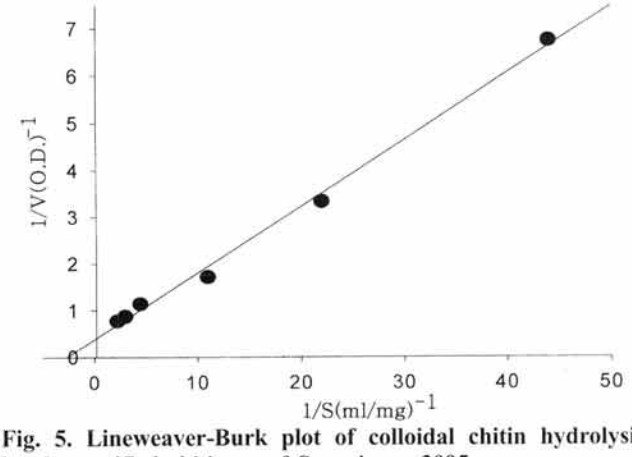

Fig. 5. Lineweaver-Burk plot of colloidal chitin hydrolysis by the purified chitinase of *Serratia* sp. 3095.

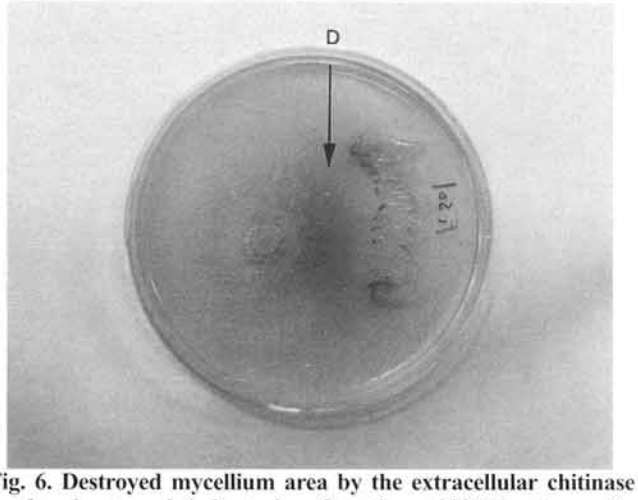

Fig. 6. Destroyed mycellium area by the extracellular chitinase produced antagonistic bacterium *Serratia* sp. 3095 (*in vitro* parir-ing culture). D; Decayed zone by produced chitinase from *Serratia* sp. 3095; Left, *Fusarium solani*; Right, *Serratia* sp.3095.

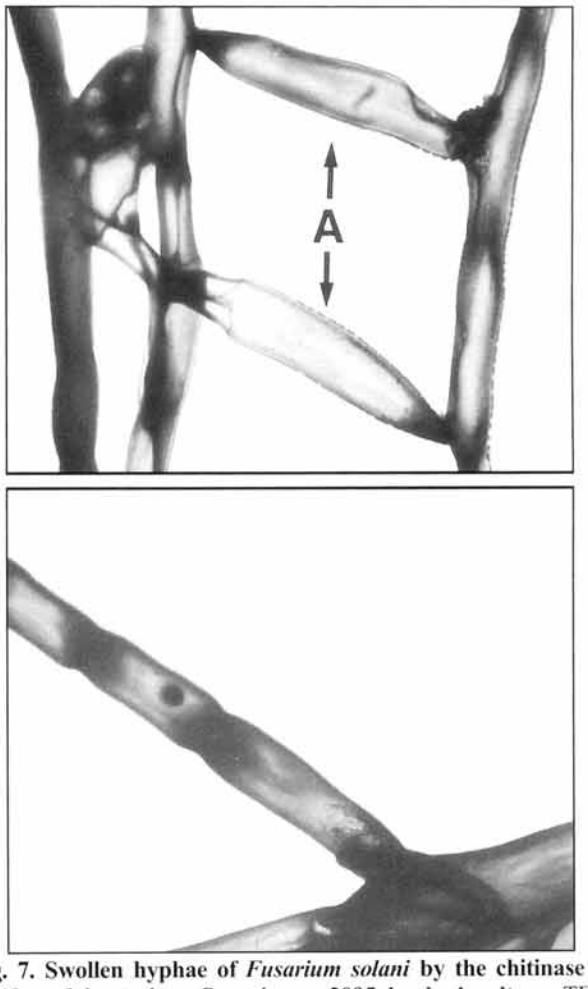

Fig. 7. Swollen hyphae of *Fusarium solani* by the chitinase of antifungal bacterium *Serratia* sp. 3095 in dual culture. TEM (×2,500). Upper: A; Swollen hyphae by chitinase. Lower; Un-treated control hyphae.

Antifungal activity by *Serratia* sp. 3095. The antifungal decayed zone of *Fusarium* mycelieum by *Serratia* sp.

3095 was investigated on the potato dextrose agar plate with a pairing culture. Inhibition zone of hyphal growth of *F. solani* was observed around the colonies of *Serratia* sp. 3095 as shown in Fig. 6. To investigate the lysed *F. solani* hyphea by the chitinase of *Serratia* sp. 3095, we incubated two strains in the mixture medium (pH 6.0, potato dextrose broth and King's B) at 28°C for 3 days with a dual culture and observed disintergrated *F. solani* hyphae by TEM (Fig. 7).

Acknowledgments. This research was supported by the Ministry of Agriculture and Forestry.

References

1. Muzzarelli, R. A. A. (1977) In *Chitn*, pp. 1-44, Pergamon Press, Oxford.
2. Knorr, D. (1991) Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.* **45**, 114-122.
3. Lee, Y. H., Bae, Y. K. and Jeong, E. J. (1996) Enzymatic hydrolysis of crystalline chitin in an agitated bead reaction system and its reaction characteristics. *J. Microbiol. Biotechnol.* **6**, 432-438.
4. Woo, C. J., Yun, U. J. and Park, H. D. (1996) Isolation of chitin-utilizing bacterium and production of its extracellular chitinase. *J. Microbiol. Biotechnol.* **6**, 439-444.
5. Lee, H. S., Lee, H. J., Choi, S. W., Her, S. and Oh, D. H. (1997) Purification and characterization of antifungal chitinase from *Pseudomonase* sp. YHS-A2. *J. Microbiol. Biotechnol.* **7**, 107-113.
6. Flach, J., Pilet, P. E. and Jolles, P. (1992) What's new in chitinase research? *Experientia* **48**, 701-716.
7. Ponstein, A. S., Bres-Vloemans, A. B., van den Elzen, P. J. M., Melcher, L. S. and Cornelissen, B. J. C. (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.* **104**, 109-118.
8. De La Cruz., Hidalgo-gallego, A., Lora, J. M., Benitez, T., Pintor-Toro, J. A. and Llobell, A. (1992) Isolation and characterization of three chitinases from *Tricoderma harzianum*. *Eur. J. Biochem.* **206**, 859-867.
9. Shin, W. C., Lee, D. S., Kim, T. H., Woo, J. H., Lee, J. M., Kim, J. G. and Hong, S. D. (1995) Isolation and characterization of *Actinetobacter* sp. WC-17 producing chitinase. *J. Microbiol. Biotechnol.* **5**, 80-86.
10. Sakai, K., Narihara, M., Kasama, Y., Wakayama, M. and Moriguchi, M. (1994) Purification and characterization of thermostable β-*N*-acetylahexoaminidase of *Bacillus stearother-mophilus* CH-4 isolated from chitin-containing compost. *Appl. Environ. Microbiol.* **60**, 2911-2915.
11. Lim, H. S. and Kim, S. D. (1994) The production and enzymatic properties of extracellular chitinase from *Pseudomonas stutzeri* YPL-1, as a biocontrol agent. *J. Microbiol. Biotechnol.* **4**, 134-140.
12. Ohta, M., Yamagami, T. and Funatsu, G. (1995) Purification and characterization of two chitinase from the leaves of pokeweed (*Phytolacca americana*). *Biosci. Biotech. Biochem.* **59**, 656-661.
13. St Leger, R. J., Cooper, R. M. and Charnley, A. K. (1986) Cuticle-degrading enzymes of entomopathogenic fungi: Regulation of production of chitinolytic enzymes. *J. Gen. Microbiol.* **132**, 1509-1517.
14. Lee, E. T. and Kim, S. D. (1998) Biological control of phytopathogenic fungi by the genetic breeding of indigenous antagonistic microorganisms. In *The Report of Reserch for the Development of Agricultural Technology*, pp. 1-9, The Ministry of Agriculture and Forestry, Korea.
15. Jeuniaux, C. (1966) *Methods Enzymol.* **8**, 644-650.
16. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**, 426-428.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
18. Peter, W. I. and Peberdy, J. F. (1997) Production and purification of a chitinase from *Ewingella americana*, a recently described pathogen of the mushroom, *Agaricus bisporus*. *FEMS Microbiol. Lett.* **157**, 189-194.
19. Rodriuez, J., Copa-patino, J. L. and Perez-Leblic, M. I. (1995) Purification and properties of a chitinase from *Penicillium oxalicum* autolysates. *Lett. Appl. Microbiol.* **20**, 46-49
20. Gal, S. W., Choi, J. Y., Kim, C. Y., Cheong, Y. H., Choi, Y. J., Lee, S. Y., Bahk, J. D. and Cho, M. J. (1998) Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. *FEMS Miocrobiol. Lett.* **160**, 151-158.
21. Jeong, E. J. and Lee, Y. H. (1995) Isolation of microorgenism producing chitinase for chitooligosaccharides production, purification of chitinase, and its enzymatic characteristics. *Kor. J. Appl. Microbiol. Biotech.* **23**, 187-196.
22. Lee, K. P., Kim, C. N., Yu, J., H. and Oh, D. H. (1990) The production and purification of chitinase from *Aeromonas salmonicida* YA7-625. *Kor. J. Appl. Microbiol. Biotech.* **18**, 599-606.
23. Lee, K. P., Choi, S. J., Moon, S. O. and Oh, D. H. (1992) Properties of activity sites of chitinase from *Aeromonas salmonicida* YA7-625. *Kor. J. Appl. Microbiol. Biotech.* **20**, 68-72.