Journal of Biochemistry and Molecular Biology, Vol. 36, No. 2, March 2003, pp. 185-189

JOURNAL OF Biochemistry and Molecular Biology

© KSBMB & Springer-Verlag 2003

Purification and Characterization of Chitinase from *Streptomyces* sp. M-20

Kyoung-Ja Kim*, Yong-Joon Yang† and Jong-Gi Kim‡

Department of Life Science, Soonchunhyang University, Asan-si 336-745, Korea

†Department of Horticultural Science, Sangmyung University, Cheonan 330-180, Korea

‡Department of Horticultural Science, Jungang University, Anseong 456-756, Korea

Received 16 September 2002, Accepted 29 October 2002

Chitinase (EC 3.2.1.14) was isolated from the culture filtrate of *Streptomyces* sp. M-20 and purified by ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography, and Sephadex G-100 gel filtration. No exochitinase activity was found in the culture filtrate. The molecular mass of the purified chitinase was 20 kDa, estimated by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and was confirmed by activity staining with Calcofluor White M2R. Chitinase was optimally active at pH of 5.0 and at 30ºC. The enzyme was stable from pH 4 to 8, and up to 40ºC. Among the metals and inhibitors that were tested, the Hg^{+}, Hg^{2+}, and *p*-chloromercuribenzoic acid completely inhibited the enzyme activity. The chitinase activity was high on colloidal chitin, chitotriose, and chitooligosaccharide. The purified chitinase showed antifungal activity against *Botrytis cinerea*, and lysozyme activity against the cell wall of *Botrytis cinerea.*

Keywords: Antifungal activity, *Botrytis cinerea*, Chitinase

Introduction

Lysis of the host structure by secretion of extracellular lytic enzymes is one of the important mechanisms that are involved in the antagonistic activity of biocontrol agents (Saksirirat and Hoppe, 1991; Mathivanan *et al.*, 1997; Kim *et al.*, 2001). Among these, chitinase (EC 3.2.1.14) plays a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal pathogens (Haran *et al.*, 1993). It affects fungal growth through the lysis of cell walls (Kunz *et al.*, 1992), hypal tips, and germ tubes (Gunarantna and Balasubramanian 1994). Many chitinases have been isolated (Han *et al.*, 2000; Baek *et al.*, 2001), and several genes that encode these enzymes have also been cloned from a variety of bacteria (Robbins *et al.*, 1992; Tsujibo *et al.*, 1993). During the last decade, chitinases have received increased attention because of their wide range of applications (Rast *et al.*, 1991; Oppenheim and Chet, 1992). The enzyme could either be used directly in the biological control on microorganisms, indirectly using purified proteins, or through gene manipulation (Oppenheim and Chet, 1992). This paper deals with the purification and antifungal activity of chitinase from *Streptomyces* sp. M-20.

*To whom correspondence should be addressed.
Tel: 82-41-530-1352; Fax: 82-41-530-1350
E-mail: kyoungjakim@hotmail.com

Materials and Methods

Chemicals The *p*-nitropheny-β-N-acetylglucosaminide, glycol chitosan, lysozyme, amylose, carboxymethyl cellulose, cellobiose, caucofluor white M2R, chitobiose, chitotriose, laminarin were purchased from Sigma Chemical Co. (St. Louis, USA). All of the other chemicals and reagents that were used were of highest grade commercially available.

Organism and cultural conditions The sample that was used for this experiment was obtained from Mongolian soil. Five grams of soil were added into 50 ml distilled water. After the sample was incubated at 30ºC for 1 h, it was spread onto a chitinase detection medium that contained the following per liter; 1 g colloidal chitin, 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 4 g NaCl, and 0.5 g $MgSO_4 \cdot 7H_2O$ that was solidified with 2% agar. Clear halos were observed around 7 colonies after incubation for 7 d at 30ºC. Among the 7 colonies, one colony that showed predominant chitinase activity was selected and grown in a chitinase production medium that contained the following per liter; 1 g colloidal chitin, 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 4 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $ZnSO_4 \cdot 7H_2O$, and 0.1 mg $MnSO_4 \cdot 7H_2O$ at 30ºC for 7 d. The culture was harvested, filtered, and centrifuged. The supernatant was used for further chitinase purification.

Purification of chitinase The proteins from the culture were precipitated by ammonium sulfate (75%). The precipitate was

collected by centrifuging at 8,000 × *g* for 20 min, and resuspended in an acetate buffer (50 mM, pH 5.0). It was dialyzed against the same buffer and freeze-dried. The sample was then loaded on a pre-equilibrated DEAE-cellulose column (2 × 20 cm), and washed with the acetate buffer. The proteins were eluted in a stepwise gradient on NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected, and the absorbance was read at 280 nm in a spectrophotometer. The fractions with chitinase activity were combined, dialyzed against the acetate buffer, and concentration by lyophilization. The concentrated sample was passed through a Sephadex G-100 column (2 × 40 cm) and eluted with the acetate buffer at the rate of 15 ml/h. The fractions of 3 ml were collected, and the absorbance and chitinase activity were measured.

Chitinase assay Colloidal chitin was used as a substrate to assay chitinase activity; 0.2 g in a 2 ml acetate buffer (50 mM, pH 5.0) was incubated with 1 ml of enzyme at 30°C for 1 h. The product was measured in 1 ml of filtrate by the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of N-acetylglucosamine per ml in 60 min. Exochitinase (N-acetyl-β-glucosaminidase) activity was assayed by mixing 0.1 ml of the aliquot of the appropriately-diluted enzyme with 0.5 ml of 1.75 mM *p*-nitropheny-β-N-acetylglucosaminide in a 50 mM acetate buffer (pH 6.0). After incubation at 30°C for 30 min, the reaction was terminated by adding 0.9 ml of 0.2 M Na_2CO_3 and *p*-nitrophenol was measured at 405 nm.

Inhibition of fungal growth by the purified-enzyme extract A spore suspension of *Botrytis cinerea* was uniformly spread on plates of PDA (potato dextrose agar). Discs that were soaked with the purified enzyme extract (5 U, 10 U) were laid on the seeded plates; the control was disc-soaked in boiled-enzyme extract. Fungal growth was observed over 2 d of incubation at 30°C.

Purity and molecular mass Homogeneity and molecular mass of the purified chitinase were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel, adopting the method of Laemmli (1970). The protein molecular markers that were used were lactalbumin (14,200), trypsin inhibitor (20,000), carbonic anhydrase (29,000), glyceraldehydes 3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000). The gel was stained in 0.15% w/v Coomassie brilliant blue R-250 in methanol-acetic acid-water (40: 10: 50, by volume) for 4 h and destained in the same solvent without the dye.

Glycol chitin synthesis Glycol chitin was obtained by acetylation of glycol chitosan (Truel, J. and Asselin, A., 1989).

Activity staining SDS-PAGE was carried out with the 12% gel that contained 0.05% glycol chitin. After electrophoresis, the gels were incubated for 2 h at 37°C in 100 mM sodium acetate buffer, pH 5.0 containing 1% (v/v) Triton X-100. The gels were then stained with 0.01% Caucofluor white M2R in 0.5 M Tris-HCl, pH 8.9, for 7 min, and destained with water. The lytic zones were photographed under the UV-transilluminator.

Properties of chitinase

Effect of pH and temperature on chitinase activity and stability The pH effect was determined by incubating the purified chitinase (10 μg as protein) at different pH levels (4-11) under standard assay conditions using colloidal chitin as the substrate. Acetate buffer (50 mM) was used for pH 3-6, phosphate buffer (50 mM) for pH 7, Tris-HCl buffer (50 mM) for pH 8-9, and glycine-NaOH buffer for pH 10-11. The enzyme stability was determined after preincubation at various pHs without the substrate for 16 h.

The optimum temperature for the chitinase activity was determined by performing the standard assay in the range of 20-80°C. Thermal stability was determined by assaying the residual chitinase activity after incubation for 1 h at the previous temperature without the substrate.

Effect of metals and inhibitors on chitinase activity The enzyme was preincubated with a 10 mM concentration of different metals and inhibitors. After 30 min, the remaining chitinase activity was measured using the standard assay. The relative inhibition of the calculated enzyme was based on the release of N-acetylglucosamine.

Activity of chitinase on different substrates The purified chitinase was incubated separately with different substrates using standard assay methods. Following incubation, the release of N-acetylglucosamine was measured. The relative activity was calculated using colloidal chitin as a control.

Lysozyme activity Lysozyme activity was determined by measuring the decrease in optical density at 660 nm. The mixture contained 1.5 ml of a *Botrytis cinerea* cell suspension (optical density of 1.7) in 50 mM phosphate buffer (pH 7.0) and 5 U of the enzyme solution. The mixture was incubated at 37°C for 30 min. (Murao *et al.*, 1990).

Results and Discussion

Purification of chitinase Table 1 summarizes the results of the purification of chitinase from *Streptomyces* sp. M-20. Extracellular chitinase from *Streptomyces* M-20 was purified to 18.9-fold. The proteins from the culture were precipitated

Table 1. Purification steps of chitinase from *Streptomyces* sp. M-20

Step	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Purification fold
Culture filtrate	425	3,078	7.24	1.0
Ammonium sulfate Precipitate	220	3,130	14.2	1.9
DEAE-cellulose	42	1,260	30.0	4.1
Sephadex G-100	6	822	137.0	6

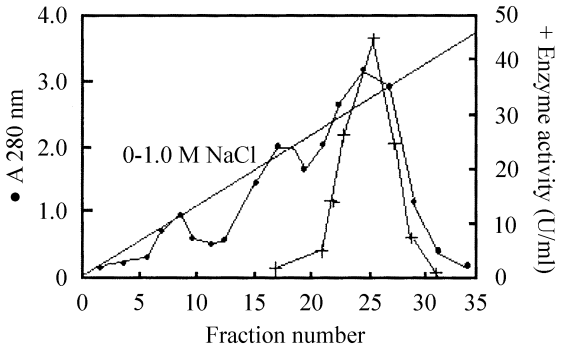

Fig. 1. Chromatogram of the chitinase from *Streptomyces* sp. M-20 on DEAE-cellulose (2.0 × 20 cm). The column was eluted with NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 3 ml were collected.

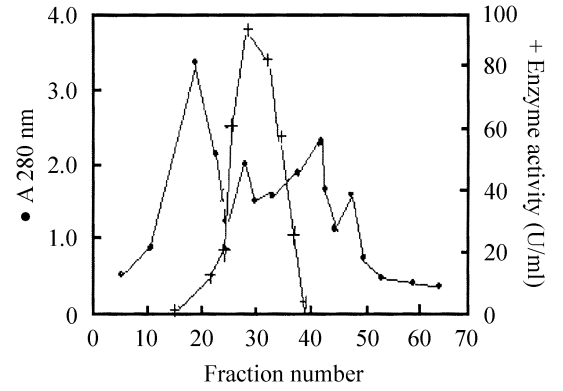

Fig. 2. Chromatogram of the chitinase from *Streptomyces* sp. M-20 on a Sephadex G-100 column (2.0 × 40 cm). The column was eluted with a 50 mM sodium acetate buffer (pH 5.0) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

by ammonium sulfate (75%). The precipitate was adsorbed by a DEAE-cellulose column, and eluted at 0.1-0.3 M NaCl in the buffer (Fig. 1). The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The elution profiles of protein and chitinase activity are shown in Fig. 2.

The purified chitinase from *Streptomyces* sp. M-20 revealed homogeneity of a single protein band on 12% native PAGE (data not shown). Its molecular weight was estimated as 20 kDa by SDS-PAGE (Fig. 3). Different molecular masses that ranged from 35-45 kDa have been reported for other fungal chitinases (Ulhoa and Peberby, 1992; Gunaratna and Balasubramanian, 1994; Sakurada *et al.*, 1996). After SDS-PAGE, the chitinase was regenerated by the removal of SDS with purified Triton X-100. The gels were stained with Calcofluor white M2R and destained. The hydrolytic zone at 20 kDa was visualized by placing the gel on a UV-transilluminator and photographed (Fig. 3).

Effect of pH The optimal pHs for chitinase activity and stability of the chitinase were examined. The enzyme was

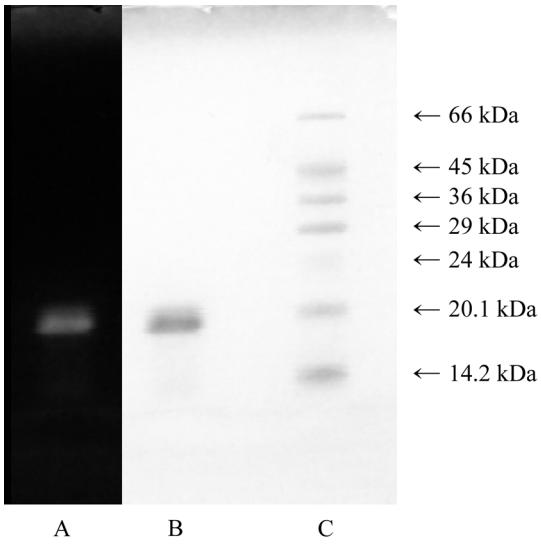

Fig. 3. SDS-PAGE of purified chitinase of *Streptomyces sp.* M-20. Activity-stained chitinase (A) and Coomassie-stained chitinase (B) and standard markers (C); lactalbumin (14,200), trypsin inhibitor (20,100), carbonic anhydrase (29,000), glyceraldehydes 3-phosphate dehydrogenase (36,000), ovalbumin (45,000), and bovine serum albumin (66,000).

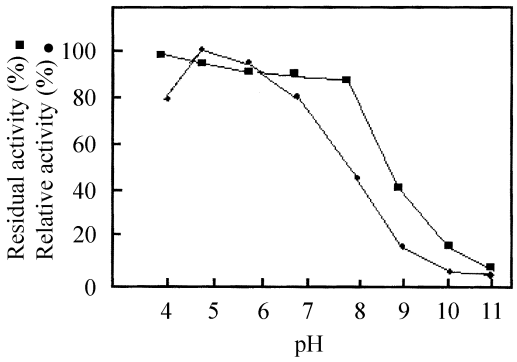

Fig. 4. Optimal pH (●) and stability of pH (■) of purified chitinase from *Streptomyces* sp. M-20.

most active between pH 5.0 and 6.0. It was relatively stable at pHs between 4.0 and 8.0, when kept at 4°C. However, beyond these pH ranges, it rapidly lost its activity (Fig. 4). Many chitinases, including the present one, showed a pH optimum in the acidic range. The *Alteromonas* sp. strain O-7 (Tsujibo, H.Y. *et al.*, 1993) was in the basic range and *Bacillus* sp. LJ-25 was in the neutral range (Lee *et al.*, 2000).

Effect of temperature The chitinase activity was most active at 30°C. Above 40°C, the activity decreased and was lost completely at 60°C. When the enzyme was kept at various temperatures for 30 min in an acetate buffer (pH 5.0), it was significantly inactivated above 50°C and completely at 65°C (Fig. 5).

Effect of metal ions and chemical reagents The inhibitory

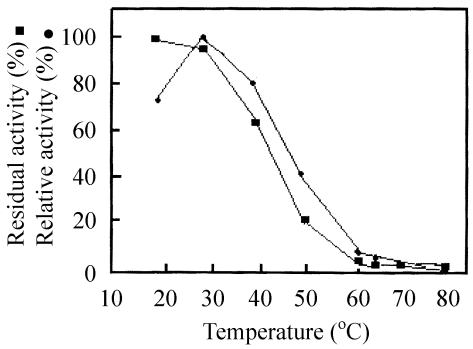

Fig. 5. Optimal temperature (●) and stability of temperature (■) of purified chitinase from *Streptomyces* sp. M-20.

Table 2. Effect of some metals and chemical reagents on chitinase activity

Material	Relative activity (%)	Material	Relative activity (%)
None	100	$CuSO_4$	96
$HgNO_3$	0	$SnCl_2$	98
$HgCl_2$	0	EDTA	90
$AgNO_3$	35	ρ-CMB	0
$CaCl_2$	95	SDS	95
$MgCl_2$	98	$FeCl_2$	80
$FeCl_3$	80	$CoCl_2$	89
$MnCl_2$	87	2-ME	170

p-CMB: *p*-chloromercuric benzoic acid
2-ME: 2-mercaptoethanol
The chitinase activities were measured at 30°C in the presence of 1 mM of various ions or reagents as the standard assay method, described in Materials and Methods.

effects of some metal ions and chemical reagents on chitinase activity were investigated in an assay system where 1 mM of each ion or reagent was present. As shown in Table 2, the chitinase activity was strongly inhibited by Ag^+ and completely by Hg^+, Hg^{2+}, and *p*-chloromercuribenzoic acid. The increase in activity with mercaptoethanol indicated the presence of sulfyhdryl groups on the active site of the enzyme, which was confirmed by the total inhibition by Hg^{2+}. Similar inhibition and mercaptoethanol enhancement was reported by Ueno *et al.* (1990).

Substrate specificity Table 3 shows the results of the hydrolysis of various substrates with the enzyme. The enzyme hydrolyzed the colloidal chitin, chitotriose, and chitooligosaccharides, but did not hydrolyze chitobiose, *p*-nitrophenyl-N-acetylglucosaminide, or any other substrate (Shon *et al.*, 2001) that was composed of glucosidic linkage. This indicates the absence of chitobiase and glycosidases, other than chitinases.

Lysozyme activity of purified-chitinase activity Lysozyme activity was determined by measuring the decrease in optical density at 660 nm. In a 50 mM phosphate buffer (pH 7.0) solution, 5U of the purified chitinase showed lysozyme activity against *Botrytis cinerea* cell suspension (optical density of 1.7). A 50% decrease in OD 660 nm was observed at 5 U of the purified chitinase from *Streptomyces* sp. M-20.

Table 3. Substrate specificity of purified chitinase of *Streptomyces* sp. M-20

Substrate	Relative activity (%)
Colloidal chitin	100
Glycol chitosan	0
p-nitropheny-β-N-acetylglucosaminide	0
Chitibiose	0
Chitotriose	250
Chitooligosaccharide*	310
Amylose	0
Carboxymethyl cellulose	0
Cellobiose	0
Laminarin	0

*Chitooligosaccharides are mixture of chitotetraose, chitopentaose, and chitohexaose.

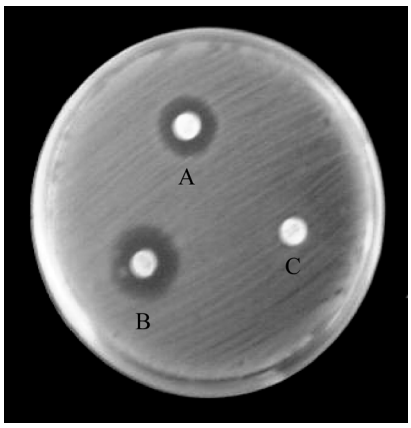

Fig. 6. Agar diffusion test with purified chitinase on *Botrytis cinerea.* The Agar diffusion test was performed on *Botrytis cinerea* agar plates with paper discs that were coated with the purified-chitinase enzyme (A, 5 U; B, 10 U enzyme; C, boiled enzyme).

Inhibition of fungal growth by purified chitinase The inhibition of the *Botrytis cinerea* growth was observed on agar plates with paper discs that were coated with the purified-chitinase enzyme (Fig. 6). Antifungal activity of the purified chitinase of *Streptomyces* sp. M-20 was confirmed. The inhibition diameter of the 10 U enzyme was greater than that of the 5 U enzyme, and the boiled enzyme had no inhibitory activity against *Botrytis cinerea*.

Acknowledgments This work was supported in part by the

2001 Research Fund from the Soonchunhyang University and Korea Science and Engineering Foundation (2000-1-22100-002-3).

References

Baek, J. H., Han, B. K. and Jo, D. H. (2001) Distribution of chitinase in rice (Oryza sativa L) seed and characterization of a hull specific chitinase. *J. Biochem. Mol. Biol*. **34**, 310-315.

Gunarantna, K. R. and Balasubramanian, R. (1994) Partial purification and properties of extracellular chitinase produced by Acremonium obclavatum, an antagonist to groundnut rust, *Puccinia arachidis. World J. Microbiol. Biotechnol*. **10**, 342-345.

Han, B. K., Moon, J. K., Ryu, Y. W., Park, Y. H. and Jo, D. H. (2000) Purification and Characterization of Acidic Chitinase from Gizzards of Broiler (Gallus gallus L.). *J. Biochem. Mol. Biol*. **33**, 326-331.

Haran, S., Schickler, H. and Chet, I. (1993) Increased constitutive chitinase activity in transformed *Trichoderma harzianum*. *Biol. Control*. *Theory Appl*. *Pest Manage*. **3**, 101-108.

Kim, S. -K., Kim, Y. -T., Byun, H. -G., Park, P. -J. and Ito, H. (2001) Purification and Characterization of antioxidative peptide from Bovine skin. *J. Biochem. Mol. Biol*. **34**, 219-224.

Kunz, C, Ludwig, A. and Boller, T. (1992) Evaluation of the antifungal activity of the purified chitinase I from the filamentous fungus *Aphanocladium album*. *FEMS Microbiol. Lett*. **90**, 105-109.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **277**, 680-685.

Lee, J. S., Joo, D. S., Cho, S. Y., Ha, J. H. and Lee, E. H. (2000) Purification and characterization of extracellular chitinase produced by marine bacterium, *Bacillus* sp. LJ-25. *J. Microbiol. Biotechnol*. **10**, 307-311.

Mathivana, N, Kabilan, V. and Murugesan, K. (1997) Production of chitinase by *Fusarium clamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*, *Indian. J. Exp. Biol*. **35**, 890-893.

Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*. **31**, 426-428.

Murao, S., Kato, M., Wang, S. L., Hoshino, M. and Shin, T. (1990) Isolation and characterization of a novel hen egg white lysozyme inhibitor from *Bacillus subtilis* I-139. *Agri. Biol. Chem*. **54**, 119-1136.

Oppenheim, A. B. and Chet, I. (1992) Cloned chitinase in fungal pathogen control strategies. *Trends in Biotechnology* **10**, 392-394.

Rast, D. M., Horsch, M., Further, R. and Goodday, G. W. (1991) A complex chitinolytic system in exponentially growing mycelium of Mucor rouxii: properties and function. *J. Gen. Microbiol.* **137**, 2707-2810.

Robbins, P. W., Overbye, K. and Pero, J. (1992) Cloning and high-level expression of chitinase-encoding gene of *Streptomyces placates*. *Gene* **111**, 69-76.

Saksirirat, W. and Hoppe, H. H. (1991) Secretion of extracellular enzymes by *Verticillium psalliotae* Treschow and *Verticillium lecanii* (Zimm.) Viegas during growth on uredospores of the soybean rust fungus in liquid cultures. *J. Phytopathol*. **131**, 161-173.

Sakurada, M., Morgavi, D. P., Komatani, K., Tomita, T. and Onodera, R. (1996) Purification and characterization of cytosolic chitinase from *Piromyces* communities OTS1. *FEMS Microbiol. Lett*. **137**, 75-78.

Shon, Y. -H., Ha, Y. -M., Jeong, T. -R., Kim, C. -H. and Nam, K. -S. (2001) Modifying Action of Chitosan Oligosaccharide on 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MelQx)-induced Mutagenesis *J. Biochem. Mol. Biol.* **34**, 90-94.

Truel, J. and Asselin, A. (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem*. **178**, 363-366.

Tsujibo, H. Okami, Y., Tanno, H. and Inamori, Y. (1993) Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol*. **175**, 176-181.

Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. (1990) Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. *J. Gen. Appl. Miciobiol*. **36**, 377-392.

Ulhoa, C. J. and Peberdy, J. F. (1992) Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. *Enzyme Microb. Technol*. **14**, 236-240.