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An Antifungal Chitinase Produced by *Bacillus cereus* with Shrimp and Crab Shell Powder as a Carbon Source

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Abstract. The production of inexpensive chitinolytic enzymes is an element in the utilization of shellfish processing wastes. In this study, shrimp and crab shell powder prepared by treating shrimp and crab processing wastes with boiling and crushing was used as a substrate for the isolation of an antifungal chitinase-producing microorganism. *Bacillus cereus* YQ 308, a strain isolated from the soil samples, excreted one chitinase when cultured in a medium containing 2% (wt/vol) shrimp and crab shell powder as major carbon source. The chitinase, purified by sequential chromatography, had an Mr of 48 kDa and pI of 5.2. The purified chitinase (2 mg/ml) inhibited the hyphal extension of the fungi *Fusarium oxysporum* and *Pythium ultimum*.

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) residues linked by β-1–4 bonds, is a common constituent of insect exoskeletons, shells of crustaceans, and fungal cell walls. The production of chitinases (EC 3.2.1.14) in higher plants may be part of their defense mechanism against infection by fungal pathogens. On the other hand, bacteria may produce chitinases for assimilation of chitin as carbon and nitrogen sources [3, 6]. It is suggested that chitinolytic microorganisms or chitinolytic enzymes have potential applications in the biocontrol of plant pathogenic fungi and insects [14, 26], as a target for biopesticides [24], and in many other biotechnological areas [19, 25]. Biological control of plant pathogens provides an attractive alternative means for management of plant disease without the negative impact of chemical fungicides that are usually costly, can cause environmental pollution, and may induce pathogen resistance.

We have previously investigated the bioconversion of shrimp and crab shell powder (SCSP) of marine waste for biofungicide production. *Pseudomonas aeruginosa* K-187, isolated from soil in Taiwan, produced both bifunctional chitinases/lysozymes extracellularly [30] and antifungal antibiotics [33]. In the present work, we found that a soil-borne strain of *Bacillus cereus* displayed chitinolytic and antifungal activities when cultured in an SCSP medium. The purification and characterization of the bacteria-produced antifungal chitinase is investigated.

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Materials and Methods

Materials. The shrimp and crab shell powder (SCSP) used in these experiments was purchased from Chya-Pau Co., I-Lan, Taiwan. Flake chitosan was from In-Hwa Co., Kau-Shion, Taiwan. DEAE-Sepharose CL-6B and Sephacryl S-200 were from Pharmacia. Ethylene glycol chitin (EGC), glycol chitin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*-NP-GlcNAc), *p*-nitrophenyl-tetra-acetyl-β-D-glucosaminide [*p*-NP-$(GlcNAc)_4$], lyophilized cells of *Micrococcus lysodeikticus*, carboxymethyl cellulose (CMC), xylan, and powdered chitin were purchased from Sigma Chemical Co., St. Louis, MO. Colloidal chitin was prepared from powdered chitin (Wako Chemicals, Japan) by the method of Jeniaux [10]. Cell suspensions of *M. lysodeikticus* were prepared as described previously [30]. All other reagents used were of the highest grade available.

Isolation and screening of chitinase-producing strains. *Bacillus cereus* YQ 308 was isolated from soils collected at different locations in Taiwan and was screened on agar plates containing 0.5% (wt/vol) shrimp and crab shell powder (SCSP), 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ and 2% (wt/vol) agar (pH 7). The plates were incubated at 30°C for 2 days.

The plant-pathogenic fungi used in this study were *Fusarium oxysporum* CCRC35100 and *Pythium ultimum* CCRC32725 from the Culture Collection and Research Center, Taiwan.

Identification of strain YQ308. The bacterial strain YQ308 (Gram-positive) was characterized from morphological observation and phys-

iological characteristics. The microorganisms were further identified according to the description in *Bergey's Manual of Determinative Bacteriology* [11] (identified by Food Industry Research and Development Institute, Taiwan).

Measurement of antifungal activity. The antifungal activity of the supernatant obtained above was estimated by using a growth inhibition assay described earlier [32]. Fungal spores of pathogenic *F. oxysporum*, *F. solani*, and *P. ultimum* were grown on Petri plates filled with potato/dextrose/agar (PDA). After 10 days' incubation at 25°C, the fungal spores were removed with sterile water containing 0.1% (vol/vol) Tween 80. The resulting suspension was filtered aseptically through sterilized gauze. The filtrate was adjusted with sterile water to a concentration of 1×10^6 spores per milliliter and was stored at 4°C. To test the antifungal inhibitory effect of the enzyme obtained above, Petri plates were filled with 5 mL of molten PDA pre-cooled to 45°C and divided into two groups (triplicate for each). To each plate in the experimental group (E), a properly diluted enzyme solution (5 mL) was added. The ratio (vol/vol) of the enzyme and PDA added in the Petri plates was 1:1. To those of the control group (C) was added an equal amount of sterile water instead of enzyme solution. After the plates were cooled, the fungal spores (20 μL) were placed onto an agar surface. Both groups were incubated for 72 h at 25°C. The diameters of the largest and smallest fungal colonies were recorded, and the averages were calculated. The inhibition ratios were calculated with the following formula.

$$\text{Inhibition ratio (\%)} = (C - E)/C \times 100\%$$

where C is the average diameter of the largest and smallest colonies of the control groups and E is the average diameter of the largest and smallest colonies of the experimental groups.

Generally, if the inhibitory ratio was greater than 20%, the tested fungus would be considered inhibited. To express the inhibitory activity of the enzyme, one unit of antifungal activity was defined as the amount of enzyme required to obtain 50% inhibition under the above assay conditions.

Chitinase production and purification. For the isolation of chitinase, *Bacillus cereus* YQ308 was grown in 100 ml medium (2% wt/vol shrimp and crab shell powder, 1% wt/vol flake chitosan, 0.1% carboxymethyl cellulose, 0.1% polypeptone, 0.2% NaCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$) in a 250-mL Erlenmeyer flask at 37°C and pH 7. Growth was monitored by determination of the cell dry weight.

Culture supernatant was collected from 3-day-old cultures by centrifugation at 12,000 *g* for 20 min. The supernatant (895 mL) was concentrated by precipitation with ammonium sulfate (608 g/L), the concentrated fraction was dialyzed against 50 mM potassium phosphate buffer (pH 7), and the enzymes were separated by DEAE-Sepharose CL-6B column chromatography followed by Sephacryl S-200 gel filtration chromatography. The protein peak fractions containing chitinase activity were collected, concentrated with ammonium sulfate, and dialyzed against 50 mM potassium phosphate buffer (pH 7).

Measurement of enzyme activity. Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1 mL of substrate solution of 1.5% (wt/vol) suspension of colloidal chitin in a phosphate buffer (50 mM, pH 7), and the mixture was incubated at 37°C for 15 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita [9] with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of the enzyme that produced 1 μmol of reducing sugar per minute. Glycol chitosanase activity was measured as an increase in reducing powder resulting from hydrolysis of glycol chitosan (in 50 mM phosphate buffer, pH 7). EGCase activity was also measured as an increase in reducing power resulting from hydrolysis of EGC (in 50 mM phosphate buffer, pH 7) at 37°C for 30 min [30]. β-*N*-acetyl-glucosaminidase and β-*N*-acetyl-hexosaminidase activities were determined by the release of *p*-nitrophenyl from *p*-NP-GlcNAc and *p*-NP-$(GlcNAc)_4$, respectively. One unit of enzyme activity was defined as 1 μmol of *p*-nitrophenol formed per milliliter of reaction mixture during 10 min at 37°C. The enzyme activities on the substrates *M. lysodeikticus*, CMC, xylan, and casein were assayed by the procedures as described previously [30, 31].

Protein content was estimated by the method of Bradford [2] with Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as standard.

Determination of molecular weight and isoelectric point. The molecular weight of the purified enzyme was determined by SDS-PAGE according to the method of Laemmli [12]. The standard proteins used for calibration were myosin (molecular weight 220,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,300). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (5:1:5, vol/vol) and decolorized in 7% acetic acid. The chitinase solution (1 mL) was loaded onto a chromatofocusing PBE 94 column (1 × 40 cm) equilibrated with 25 mM histine/HCl buffer (pH 6.2), and the elution was done with Polybuffer 74/HCl (pH 4.0) as described in the manufacturer's manual (Pharmacia).

Analysis of the oligosaccharides by HPLC. The compositions of the oligosaccharides obtained by enzymatic hydrolysis were analyzed by HPLC on TSK-GEL G-OLIGO-PW column (7.8 × 300 mm, TOSOH Co. in Japan) at 50°C. Then, 20 μL of sample was chromatographed with distilled water (L.C. Grade) as the elution and a flow rate 0.8 mL/min. The oligosaccharides were detected by monitoring the refractive index.

Effect of YQ308 chitinase on growth of plant-pathogenic fungi. *Fusarium oxysporum* and *Pythium ultimum* were grown separately in 20 mL potato/dextrose/broth (PDB) in 250-mL flasks. The culture flasks were incubated on an orbital shaker at 150 rpm and 30°C. Sterilized chitinase solution, 2 mg/mL, was added to each culture, which was grown for 0, 6, 12, or 24 h and incubated for 48 h at 30°C with appropriate controls. The control group received an equal amount of sterile water instead of enzyme solution. The experiments were run in triplicate and repeated two times. The dry weight of the mixed culture was assessed after filtration through pre-weighted Whatman No. 1 filter paper.

Results

Identification of strain YQ308. From the morphological observation and physiological characteristics (data not shown), the strain YQ308 was identified as a strain of *Bacillus cereus*.

Purification of YQ308 chitinase. When grown on SCSP as a major carbon source, *Bacillus cereus* YQ308 released chitinase into the culture fluid with the highest activity at 2.5–3.5 days (Fig. 1). The purified chitinase (see Table 1) was confirmed to be homogeneous by SDS-PAGE (Fig. 2) and chromatofocusing (data not

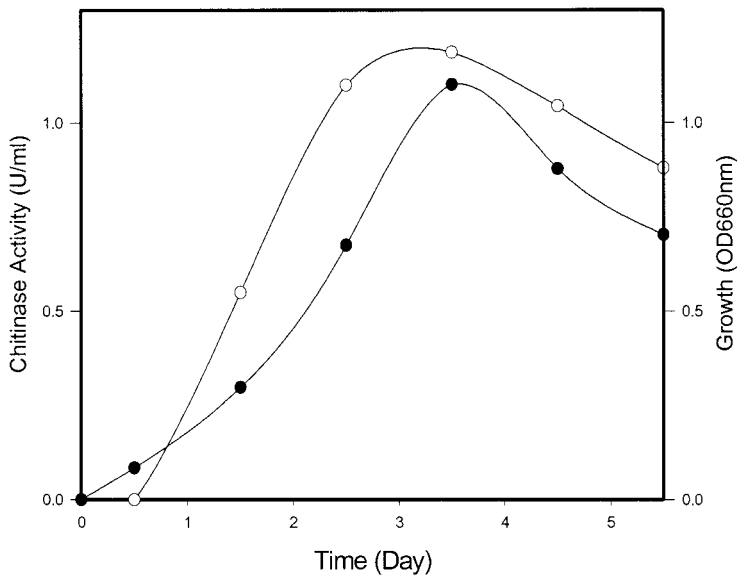

Fig. 1. Time courses of growth (●) and chitinase activity (○) in a culture of *B. cereus* YQ308. The chitinase activity was highest at 2.5–3.5 days.

Table 1. Purification of the chitinolytic and antifungal activity of *B. cereus* YQ308 chitinase[a]

	Purification step			
	Culture sup.	$(NH_4)SO_4$ ppt	DEAE-Sepharose CL-6B	Sephacryl S-200
Total protein (mg)	1136	728	96	16
Chitinase activity:				
Total activity (U)	983	753	522	151
Sp. act. (U/mg)	0.9	1.1	5.4	9.5
Yield (%)	100	77	53	15
Anti-FO activity:				
Total activity (U)	1476	407	30	6
Sp. act. (U/mg)	0.13	0.06	0.03	0.04
Yield (%)	100	27.6	2.0	0.4
Anti-FS activity:				
Total activity (U)	5020	1350	81	21
Sp. act. (U/mg)	0.44	0.14	0.10	0.13
Yield (%)	100	27.0	1.6	0.4

[a] *Bacillus cereus* YQ308 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 2% (wt/vol) shrimp and crab shell powder, 1% (wt/vol) flake chitosan, 0.1% carboxymethyl cellulose, 0.1% polypeptone, 0.2% NaCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ in a shaking incubator for 3 days at 37°C.

shown). The molecular weight of the chitinase was estimated by SDS-PAGE to be 48 kDa, and the pI of the chitinase was 5.2 by chromatofocusing.

YQ308 chitinase displayed antifungal activities against the plant-pathogenic fungi *F. oxysporum* (FO) and *F. solani* (FS). The purification steps give overall purification of 10.6-fold for chitinase activity against colloidal chitin, 0.30-fold for antifungal activity against FO (anti-FO), and 0.31-fold for antifungal activity against FS (anti-FS). The activity yields of the purified chitinase were 15% for chitinase activity and 0.4% for both activities of anti-FO and anti-FS. The specific activities of chitinase, anti-FO, and anti-FS were 9.5, 0.13, and 0.04 U/mg of protein, respectively.

Enzymatic activity. The purified chitinase was assayed with various substrates, i.e., 1% colloidal chitin (chitinase activity), 0.1% ethylene glycol chitin (EGCase activity), 0.1% glycol chitosan (chitosanase activity), 5mM *p*-NP-(GlcNAc) (β-*N*-acetyl-glucosaminidase activity), 5mM *p*-NP-$(GlcNAc)_4$ (β-*N*-acetyl-hexosaminidase activity), *M. lysodeikticus* cells (lysozyme activity), 1.25% CMC (cellulase activity), 0.5% xylan (xylanase activity), and 1% casein (protease activity). YQ308 chitinase showed higher hydro-

Fig. 2. SDS-PAGE of YQ308 chitinase. Right S, molecular weight markers (top to bottom: 220 kDa, 97 kDa, 66 kDa, 45 kDa, 30 kDa, 20.1 kDa, 14.3 kDa). Left I, purified YQ308 chitinase.

lyzing activity against colloidal chitin (5.6 U/mg) and ethylene glycol chitin (7.7 U/mg), and little activity against glycol chitosan (0.5 U/mg). There was no enzymatic activity on *p*-NP-GlcNAc, *p*-NP-$(GlcNAc)_4$, *M. lysodeikticus* cells, CMC, xylane, or casein.

To further clarify the cleavage, 3% of colloidal chitin was hydrolyzed by YQ308 chitinase for 24 h, and the reaction products were analyzed by using HPLC. As shown in Fig. 3, $(GlcNAc)_4$ is the major hydrolyzing product.

Effects of pH and temperature. Using colloidal chitin as a substrate under the standard assay conditions, maximum activity of the purified chitinase was at pH 7. It was stable at pH 6–9 for 30 min. The optimum temperature for chitinase activity was 50°C over 10 min of incubation. It lost 60% of its activity at 80°C over 10 min and was completely inactivated at 100°C incubated for 10 min.

Effects of various chemicals. The chitinase, when incubated in 50 mM phosphate buffer (pH 7) for 10 min at 37°C, was completely inactivated by 1 mM Hg^{2+}. Other inorganic ions had less inhibitory effect.

Effect of YQ308 chitinase on growth of plant-pathogenic fungi. A significant reduction in the biomass of test fungi resulted following co-incubation with the purified chitinase after 24 h compared with test fungi grown alone in PDB medium. With the increase in growth time of the fungal culture there was a decrease in inhibition (Fig. 4).

Discussion

Bacillus cereus YQ308, isolated from the soil samples, released antifungal enzymes into the culture broth when it was grown aerobically in the SCSP-containing medium. One antifungal chitinase isolated and purified from the culture broth displayed antifungal activity on plant-pathogenic fungi *F. oxysporum*, *F. solani*, and *P. ultimum*. The purity of the enzyme after purification was supported by the results of SDS-PAGE and chromatofocusing performed for the measurement of the molecular weight and isoelectric point. As with the *B. cereus* strain 65 [20], we detected only one chitinase in the culture supernatant of *B. cereus* YQ308. Although four distinct chitinases have been purified from *B. cereus* CH, the difference of their molecular sizes may be caused by proteolytic modification of their carboxyl terminal regions [16]. In our preliminary experiment, we found the enzyme activity of YQ308 chitinase was not affected by the protease partially purified from the culture supernatant of *B. cereus* YQ308.

The molecular weight of YQ308 chitinase is approximately 48,000 by SDS-PAGE, which is apparently different from those of known *Bacillus* chitinases. For example, 36,000 for *Bacillus cereus* 6E1 [34]; 36,000 for *B. cereus* 65 [20]; 35,000, 47,000, 58,000, 64,000 for *Bacillus cereus* CH [16]; 68,000, 38,000, 52,000 for *Bacillus cereus* VKPM B-6838 [29]; 74,000, 69,000, 38,000, 38,000, 39,000, 52,000 for *Bacillus circulans* WL-12 [35, 36]; 41,000 for *Bacillus* sp. BG-11 [1]; 35,000, 46,000 for *Bacillus* sp. X-b [7]; and 71,000, 62,000, 53,000 for *Bacillus* sp. MH-1 [20].

Most of the bacterial chitinases have acidic pIs, and actinomyces chitinases have neutral or alkaline pIs. Plant chitinases generally have very basic or very acidic isoelectric points. *B. cereus* YQ308 chitinase has similar acidic pI (pH 5.2) as the other bacterial chitinases, but it is different from the other reported *B. cereus* chitinase (pH 6.4) [20].

When colloidal chitin was used as a substrate for measuring chitinase activity, the optimum pH for YQ308 chitinase was 7. Other *B. cereus* chitinases also work better at an acidic or near neutral pH. For example, *B. cereus* 6E1 is at 5.8 [34], and *B. cereus* CH is at 5.0–7.5 [16]. The optimum temperature of YQ308 chitinase differs from those observed for chitinases of other *B. cereus* origins [16, 34].

On the basis of catalytic mechanisms, chitinases catalyzing the hydrolysis of 1,4-β-D-glycosidic bonds linking the *N*-acetylglucosamine subunits of chitin are classified into three groups consisting of the endochitinases and two exochitinase groups, i.e., chitobiosidases and β-*N*-acetylhexosaminidase [3, 4, 34]. Endochitinases cleave chitin randomly, generating soluble, low-molecular-weight GlcNAc multimers, such as chitobiose, chitotriose, and chitotetraose. Chitobiosidases cleave chitin to release GlcNAc dimers (chitobiose) one at a time from the non-reducing end of the chitin chain. β-*N*-acetyl-

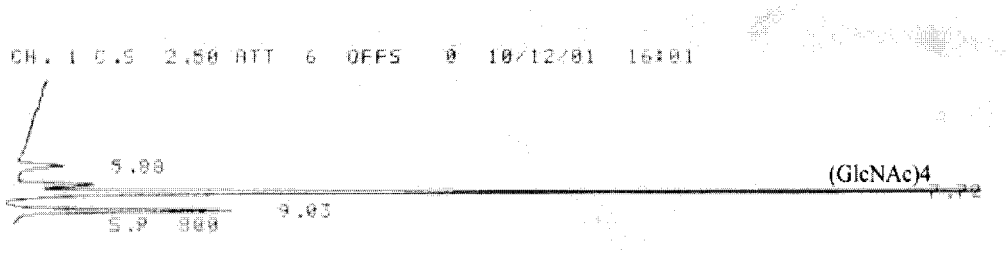

Fig. 3. HPLC analysis of the reaction products from colloidal chitin after hydrolysis with YQ308 chitinase.

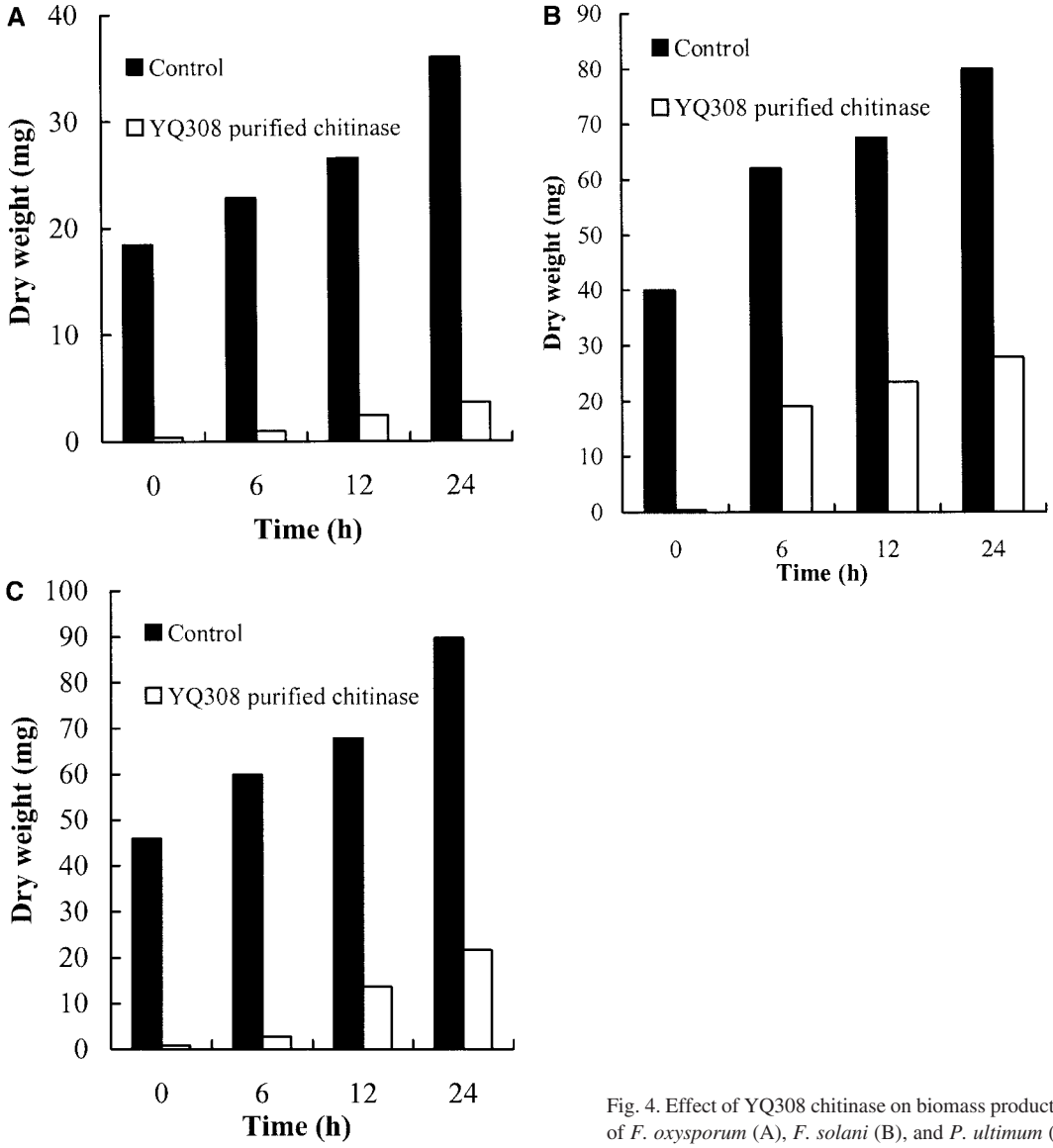

Fig. 4. Effect of YQ308 chitinase on biomass production of *F. oxysporum* (A), *F. solani* (B), and *P. ultimum* (C).

hexosaminidases hydrolyze chitobiose, chitotriose, and chitotetraose from the non-reducing end of *N*-acetylchitooligosaccharides, resulting in the release of *N*-acetylglucosamine [34]. According to this nomenclature sys-

tem, YQ308 chitinase is not a β-*N*-acetylhexosaminidase because it does not hydrolyze *p*-NP-GlcNAc. It could be an endochitinase, since it catalyzes release of *p*-NP-$(GlcNAc)_4$ (chitotetraose) from colloidal chitin.

The main function of bacterial extracellular hydrolases is, undoubtedly, the release of nutrients from different substrates for the needs of a bacterium. In addition, excreted enzymes alone, or with other compounds like antibiotics, may be used by bacteria for competition with other microbial species. Numerous microorganisms with antifungal activities have been identified, and many have been effective in field experiments. So far, bacteria, especially *Pseudomonas* strains [8, 13, 17, 27, 28] and *Bacillus* strains [7], have been intensively investigated as biological control agents.

Roberts and Selitrennikoff [21, 22] studied plant and bacterial chitinases for antifungal activity and enzyme specificity. According to their results, plant chitinases isolated from the grains of wheat, barley, and maize functioned as endochitinases and inhibited hyphal elongation of test fungi. In contrast, bacterial chitinases from *S. marcescens*, *S. griseus*, and *Pseudomonas stutzeri* acted as exoenzymes and had no effect on hyphal extension of test fungi like *T. reesei* and *Phycomyces blakesleeanus*. Pleban et al. [20] found the crude extracellular chitinase of an endophytic bacterium *B. cereus* 65 decreased spore germination of *F. oxysporum*. Ordentlich et al. [18] found crude chitinase of *Serratia marcescens* caused lysis of hyphal tips of *Sclerotium rolfsii*. Shapira et al. [26] found partially purified chitinase of *S. marcescens* to be effective in reduction of disease incidence caused by *S. rolfsii*. We observed that *B. cereus* YQ308 chitinase inhibited the growth of plant-pathogenic fungi *F. oxysporum*, *F. solani*, and *P. ultimum*. Similar phenomena were seen when plant-pathogenic fungi were treated with purified fungal endochitinase from *Trichoderma harzianum* [14, 15] and *Gliocladium virens* [5]. Combine the result with $(GlcNAc)_4$ as the major hydrolyzing product, it is further confirmed that YQ308 chitinase may act as endochitinase, and the inhibition of growth of test fungi presumably attribute to the action of endochitinase. Therefore, the antifungal activity of YQ308 chitinase seems to be similar to that of chitinases of fungi and plants.

In summary, the characteristics of YQ308 chitinase differ from the known *B. cereus* chitinases in terms of their molecular weights and isoelectric points. Compared with other known bacterial chitinases, the unique characteristics of YQ308 chitinase include antifungal activity against plant-pathogenic fungi and production of chitotetraose as the major enzymatic hydrolyzate from colloidal chitin. To determine the mechanism of induction and the function of emerging YQ308 chitinase in the degradation process of chitin and plant-pathogenic fungal cells, we need to know the nucleotide sequence of the gene. Cloning of the YQ308 chitinase is now under way.

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