World J Microbiol Biotechnol (2009) 25:375–382
DOI 10.1007/s11274-008-9901-5

ORIGINAL PAPER

Conversion of crude chitosan to an anti-fungal protease by *Bacillus cereus*

Wen-Teish Chang · Cheng-Hong Hsieh · Hung-Sheng Hsieh · Chinshuh Chen

Received: 20 February 2008 / Accepted: 25 October 2008 / Published online: 16 November 2008

Abstract *Bacillus cereus* AU004, isolated from soil samples, secreted a complex of hydrolytic enzymes into the culture broth when it was grown aerobically in a medium containing crude chitosan flakes. The presence of the AU004 culture supernatant substantially influenced the growths of the plant-pathogenic fungi *Fusarium oxysporum*, *F. solani* and *Pythium ultimum* in terms of dry weight. AU004 excreted a protease when cultivated in a medium that contained 4% (w/v) chitosan as the major nutritional source. The protease was purified by sequential chromatography and characterized as a novel extracellularly neutral protease. The protease had an Mr of 28.8 kDa. The optimal pH and temperature for protease activity were 7 and 50°C, respectively. Antifungal activity of the protease was observed using an assay based on the inhibition of spore germination and hyphal extension of the fungal *Pythium ultimum*. This investigation is the first report of the production of an anti-fungal protease from *Bacillus* spp.

Keywords Antifungal · *Bacillus cereus* · Chitosan · Protease · *Pythium ultimum*

C. Chen (✉)
Department of Food Science and Biotechnology, National ChungHsing University, 250, Kuokuang Rd., Taichung, Taiwan, ROC
e-mail: chinshuh@dragon.nchu.edu.tw

W.-T. Chang · C.-H. Hsieh · H.-S. Hsieh
Department of Health and Nutrition Biotechnology, Asia University, Lioufeng, Wufeng 500, Lioufeng Rd., Taichung County, Taiwan, ROC

Introduction

Chemical fungicides continue to have a central role in current farming practices for protecting crops against diseases. However, their use has come under increased scrutiny in recent years, since several of them are very toxic; can cause severe environmental problems; leave fungicide residues in food products, and induce pathogen resistance. These limitations have led to an intensive search for alternative control strategies, and biological methods of controlling plant pathogens provide an environmentally friendly alternative to chemical pesticides. Accordingly, biological control tactics have become an important technique for facilitating sustainable agriculture (Lindow et al. 1983).

Biological control of plant pathogens is based on the negative interaction between pathogens and biocontrol agents. One of the strategies involves the use of beneficial bacteria that are antagonistic to the harmful microorganisms (Lindow et al. 1983; Lim et al. 1991; Bagnasco et al. 1998; Moenne-Loccoz et al. 1998; Natsch et al. 1998). *Bacillus cereus* UW85 accumulates two antibiotics, zwittermicin A and kanosamine, in its culture supernatant. Zwittermicin A is a water-soluble, acid-stable linear aminopolyol molecule with broad-spectrum activity against numerous fungal and bacterial plant pathogens (He et al. 1994). Alternatively, plant pathogens can be controlled by exposing them to lytic enzymes such as chitinases, chitosanases, proteases or glucanases (Lorito et al. 1993; Harman et al. 1993; Mahadevan and Crawford 1997; Dunne et al. 2000; Janice and Carlos 2002; Wang et al. 2002a; Leelasuphakul et al. 2006; Gao et al. 2008) or antibiotics (Thomashow and Weller 1988). Genetic engineering to increase the amount of these metabolites can result in substantial improvement in or greatly improve their biocontrol activity (Flores et al. 1997; Dunne et al. 2000).

Bioconversion of marine crustacean wastes to generate biofungicide has been recently examined (Wang et al. 1999; Wang et al. 2002a; Wang et al. 2002b; Chang et al. 2007). In this investigation, a soil-borne strain of *Bacillus cereus* exhibited antifungal protease activity when cultivated in a flake-crude-chitosan (protein -containing) medium. The effects of the antifungal compounds on spore germination and germ spore elongation of pathogenic *Pythium ultimum* were investigated. The purification and characterization of the antifungal protease from the bacterium were also studied.

Materials and methods

Materials

Crude chitosan flakes were obtained from In-Hwa Co. (Kaohsiung, Taiwan). Shrimp and crab shell powder (SCSP) used in these experiments was purchased from Chya-Pau Co. (I-Lan, Taiwan). DEAE-Sepharose CL-6B and Sephacryl S-200 were purchased from Pharmacia Co. (Peapack, NJ, USA). Ethylene glycol chitin (EGC), 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 (Lindow et al. 1983). Cell suspensions of *M. lysodeikticus* were prepared as described elsewhere (Wang et al. 1997). All other reagents used were of LC grade.

Effect of culture conditions

Bacillus cereus AU004 was isolated from soil in Taiwan and maintained on nutrient agar plates at 30°C. Cultures were grown in basal medium that contained 0.1% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ (pH 7), and gradually supplemented with the various nutritional sources to be studied in turn. The major nutritional sources of interest herein are SCSP, chitin, chitosan, Chinese herb, tea leaves and cellulose. They were added at concentrations of 1%, 2%, 3%, 4% and 5% (w/v). One hundred ml of the resultant medium in a 250 ml Erlenmeyer flask was aerobically cultivated at 30°C for 24 h in a rotary shaker (150 rpm). The supernatant obtained following centrifugation at 8000*g* and 4°C for 20 min was used for bioassay. An earlier effective experiment was used as the basis for the subsequent experiment until the optimal medium condition was reached. Given the optimal culture composition, effects of the initial pH (4, 5, 6, 7, 8, and 9), temperature (25, 30, 37, and 45°C), culture volume (25 ml, 50 ml, 100 ml, 150 ml, 200 ml) and cultivation time (12, 24, 36, 48, 60, 72, and 84 h) on the production of antifungal compounds were studied.

The plant-pathogenic fungi used herein in this work were *Fusarium oxysporum* CCRC35100, *F. solani* CCRC32511 and *Pythium ultimum* CCRC32725. These strains were purchased from the Culture Collection and Research Center (CCRC), Taiwan.

Identification of strain AU004

The bacterial strain AU004 (gram positive) was characterized from morphological observations and the physiological characteristics. The microorganisms were further identified based on the description in Bergey's *Manual of Determinative Bacteriology* (Krieg and Holt 1984) (identified by the Food Industry Research and Development Institute, Taiwan).

Preparation of crude antifungal compounds

Bacillus cereus AU004 was cultivated under optimal culture conditions. Following centrifugation at 8000*g* and 4°C for 20 min, the supernatant was precipitated by 80% saturation of ammonium sulfate (561 g/l). The resultant precipitate was collected and dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and then dialyzed overnight against the same buffer. The resultant dialyzate was filtered aseptically through 0.45 μm-pore-size membrane filters and used in the bioasssays.

Measurement of antifungal activity

The antifungal activity of the supernatant obtained was estimated using the growth inhibition assay that has been described elsewhere (Wang and Chang 1997). Fungal spores of pathogenic *F. oxysporum*, *F. solani* and *P. ultimum* were grown on petri plates containing potato dextrose agar (PDA). After ten days of incubation at 25°C, the fungal spores were removed using sterile water that contained 0.1% (v/v) Tween 80. The resultant suspension was filtered aseptically through a sterilized gauze. The concentration of the filtrate was adjusted by adding sterile water to 1×10^6 spores per ml, and stored at 4°C. To test the antifungal activity of the enzyme obtained above, Petri plates that contained 5 ml of molten PDA pre-cooled to 45°C were divided into two groups (each in triplicate). A properly diluted enzyme solution (5 ml) was added to each plate in experimental group (E). The ratio (v/v) of the enzyme to PDA in the petri plates was 1:1. To the control group (C) an equal amount of sterile water, rather than the enzyme solution, was added. The plates were then cooled and the fungal spores (20 μl) were spread onto the agar surface. Both groups were incubated at 25°C for 72 h. The

diameters of the largest and smallest fungal colonies were measured and the averages calculated. The inhibition ratios were calculated using the following formula.

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

C: mean diameter of the largest and smallest colonies of the control groups
E: mean diameter of the largest and smallest colonies of the experimental groups

When the inhibitory ratio exceeded 20%, the test strain was regarded as inhibited and the minimal inhibitory concentration (MIC) for that strain was then determined. To express the inhibitory activity of the enzyme, one unit of antifungal activity was defined as the amount of enzyme required to cause a 50% of inhibition under the aforementioned assay conditions.

Measurement of enzyme activities

Chitinase activity was measured using colloidal chitin as a substrate. The enzyme solution (0.5 ml) was added to 1 ml of 1.5% (w/v) suspension of colloidal chitin in a phosphate buffer (50 mM, pH 7), and the mixture was incubated at 37°C for 15 min. Following centrifugation, the amount of reducing sugar that was generated in the supernatant was determined by the method of Imoto and Yagishita (1971) with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of the enzyme that generated 1 µmole of reducing sugar per min. Glycol chitosanase activity was measured as an increase in reducing power that was caused by hydrolysis of glycol chitosan (in 50 mM phosphate buffer, pH 7). EGCase activity was also measured as an increase in reducing power due to the hydrolysis of EGC (in 50 mM phosphate buffer, pH 7) at 37°C for 30 min. *β-N*-acetyl-glucosaminidase and *β-N*-acetyl-hexosaminidase activities were determined by the release of *p*-nitrophenol from *p*-NP-GlcNAc and *p*-NP-$(GlcNAc)_4$, respectively. One unit of enzyme activity was defined as the formation of 1 µmol of *p*-nitrophenol per ml of reaction mixture in 10 min at 37°C. The enzyme activities on the substrates *M. lysodeikticus*, CMC, xylan, and casein were assayed following the procedures described above.

To assay the protease activity, a diluted enzyme solution (0.2 ml) was mixed with 2.5 ml of 1% (w/v) casein in phosphate buffer (pH 7) and incubated at 37°C for 10 min. The reaction was terminated by adding 5 ml of 0.19 M trichloroacetic acid. The reaction mixture was centrifuged and the soluble peptide in the supernatant was measured using the method described by Todd using tyrosine as the reference compound (Scher and Baker 1982).

Determination of molecular weight

The molecular weight of the purified protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The standard proteins used in the calibration were phosphorylase b (molecular weight = 97,000 Da), albumin (67,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (20,100 Da) and α-lactalbumin (14,400 Da).

Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) that contained 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (5:1:5, v/v) and decolorized in 7% acetic acid.

Enzyme production and purification

To produce protease, *Bacillus cereus* AU004 was grown in 50 ml medium that contained 4% (w/v) crude chitosan, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ in a 250 ml Erlenmeyer flask at 25°C and pH 7.

The supernatant was collected from one day old cultures by centrifugation at 8000*g* for 20 min, and then was concentrated by ammonium sulfate precipitation (561 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 fractions that contained protease activity were collected, concentrated by ammonium sulfate precipitation, and dialyzed against 50 mM potassium phosphate buffer (pH 7).

Protein assay

Protein concentration was determined by the method of Lowry using bovine serum albumin (BSA) as a standard.

Effect of *B. cereus* AU004 on the growth of plant-pathogenic fungus

Fusarium oxysporum, F. solani, and *P. 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. The sterilized supernatant and purified protease solution were filtered aseptically through 0.45 µm-pore-size membrane filters before being added to cultures that had grown for 0, 6, 12, and 24 h. The cultures were then incubated at 30°C for 48 h using suitable controls. The control groups contained the same amount of sterile water as of enzyme solution that was used in the other groups. The experiments were performed in triplicate. The dry weight of the mixed culture

was evaluated following filtration through pre-weighed Whatman No. 1 filter paper.

Effect of *B. cereus* AU004 protease on spore germination of plant-pathogenic fungi

An aliquot of 0.25 ml fungal spores (10^6/ml) of *F. oxysporum, F. solani* and *P. ultimum*, 0.5 ml of potato dextrose broth, and 0.25 ml of purified protease were placed in eppendorf tubes. The resultant mixture was incubated at 30°C, and the spores were observed under a light microscope at various intervals.

Results

Effect of culture conditions on enzyme production

The results of a study on the effect of various nutritional sources on the production of protease by *B. cereus* AU004 demonstrated that chitosan was a better inducer than other nutritional sources of the production of protease and of antifungal activity (data not shown). Varying concentrations (1–5% w/v) of chitosan flakes were used and the resultant protease activity was studied. A 4% concentration of chitosan was found to be optimal. Hence, 4% chitosan was adopted to investigate the enzyme production kinetics of the tested strain. Protease activity was higher after incubation of 24 h. However, the activity declined as the culture was incubated for over 24 h. Incubation at four temperatures (25, 30, 37, and 45°C) using 4% chitosan revealed that the optimal temperature was 25°C. Also, the optimal pH of the cultivation medium was 7.

As a summary of the above results, the optimal culture conditions were aerobic growth in 50 ml of liquid medium that contained 4% flake chitosan, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, at pH 7 in an Erlenmeyer flask (250 ml), on an orbital shaking incubator for 24 h at 25°C.

Enzymatic activity

Several enzymatic activities of culture supernatant following centrifugation were assayed with various substrates, including 1% colloidal chitin (chitinase activity), 0.1% ethylene glycol chitin (EGCase activity), 0.1% glycol chitosan (chitosanase activity), 5 mM *p*-NP-(GlcNAc) (*β-N*-acetyl-glucosaminidase activity), 5 mM *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). Therefore, the culture supernatant exhibited hydrolyzing activity against colloidal chitin (0.82 U/mg), ethylene glycol chitin (1.2 U/mg) and casein (0.55 U/mg), and an even greater activity against glycol chitosan (2.65 U/mg). However, no enzymatic activity occurred on; *p*-NP-GlcNAc, *p*-NP-$(GlcNAc)_4$, *M. lysodeikticus* cells, CMC or xylan (Table 1).

Table 1 Activities of enzyme from culture supernatant of *B. cereus* AU 004 on various substrates

Substrates	Conc.	Enzyme activity (U mg^{-1})[a]
Colloidal chitin	1%	0.82
Ethylene glycol chitin	0.1%	1.2
Glycol chitosan	0.1%	2.65
CM-cellulose	1.25%	n.d.
xylan	0.5%	n.d.
p-nitrophenyl-*N*-acetyl-glucosaminide	5 mM	6×10^{-4}
p-nitrophenyl-Tetraacetyl-glucosaminide	5 mM	3×10^{-5}
Casein	1%	0.55
Starch	1.5%	n.d.

[a] The assay conditions used for determinations of enzymatic activities see Materials and Methods

The purified enzyme exhibited both protease (2.7 U/mg) and antifungal (1.5 U/mg) activities no other enzyme activity that was tested for.

Purification of protease

The purified protease (Table 2) was verified to be homogeneous using SDS-PAGE (Fig. 1). The molecular weight of the protease was estimated to be 28.8 kDa.

AU004 protease exhibited only antifungal activities against the plant-pathogenic fungus *P. ultimum*. The purification steps yield 4.9-fold overall protease activity, and 3.4-fold better antifungal activity against *P. ultimum*. The activity yields of the purified protease were 3% for protease activity and 2% for antifungal activity against *P. ultimum*. The specific activities of protease and antifungal activity against *P. ultimum* were 2.7, and 1.5 U/mg of protein, respectively.

Effects of pH and temperature

When casein was used as a substrate under the standard assay conditions, the maximum activity of the purified protease was observed at pH 7, while the optimum temperature for protease activity was 50°C for 10 min of incubation.

Effect of *B. cereus* AU004 on growth of plant-pathogenic fungi

The biomass of the tested fungi resulted were significantly lower when they were incubated with culture supernatant

Table 2 Proteolytic and antifungal activities of protein from *Bacillus cereus* AU004 at various purification steps[a]

Purification steps	Total protein (mg)	Protease activity				Antifungal activity			
		Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Culture sup.	1,893	1,039	0.55	1	100	837	0.44	1	100
$(NH_4)_2SO_4$ precipitate and dialysis	1,213	676	0.56	1	65	636	0.52	1.2	76
DEAE-Sepharose CL-6B	30	53	1.8	3.3	5	25.8	0.86	2	3
Sephacryl S-200	12.2	33	2.7	4.9	3	18.2	1.5	3.4	2

[a] *B. cereus* AU004 was grown in 50 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 4% (w/v) flake chitosan, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ in a shaking incubator for 1 day at 25°C

Fig. 1 SDS-PAGE of *B. cereus* AU004 protease. Left S, molecular weight markers (top to bottom: 97, 66, 45, 30, 20.1, 14.4 kDa). Right I, purified AU004 protease

for 24 h than when they were grown alone in PDB medium (Fig. 2). However, the purified protease only exhibited antifungal activities against *P. ultimum*, activities against other fungi were much weaker (Fig. 3). The inhibition decreased as the growth time of the fungal culture increased.

Effect of *B. cereus* AU004 protease on spore germination of plant-pathogenic fungi

Purified protease from *Bacillus cereus* AU004, spore suspension of *F. oxysporum, F. solani*, and *P. ultimum*, and potato dextrose broth were mixed in a ratio of 1:1:2 (v/v/v) and incubated at 25°C for 24 h. The results indicate that the antifungal activity from *B. cereus* AU004 not only reduced the spore germination of *P. ultimum* but also inhibited its elongation (Fig. 4), while the inhibitory effect on the other two fungi was much weaker. To further investigate the relationship between protease and antifungal activity from *B. cereus* AU004, the antifungal mechanism of the protease purified from *B. cereus* AU004 was studied under a light microscope. In the control group, which was incubated at 25°C for 72 h, the mycelium was intact and of uniform thickness; however, for spores of the experimental group (in which the medium, the spore suspension and the purified protease were mixed in a volume ratio of 2:1:1), *P. ultimum*, germinated. Additionally, even when germination occurred, the germ tubes were thinner than normal, and no spore was formed. However, neither hyphae cytolysis nor tail-end expansion were observed.

Discussion

Bacillus cereus AU004 secretes a complex of hydrolytic enzymes, dominated by chitinase, chitosanase and protease, and produces antifungal enzymes when it is grown in a medium that contains crude chitosan flakes of marine waste. The presence of the AU004 culture supernatant significantly affected the growths of the plant-pathogenic fungi *F. oxysporum*, *F. solani* and *P. ultimum*. The protease purified from the culture broth exhibited antifungal activity toward *Pythium ultimum*.

Recent in vitro studies have established that the exposure of selected plant-pathogenic fungi to lytic enzymes such as chitinases, proteases, cellulases or glucanases can degrade the structural matrix of fungal cell walls. Notably, neither chitinases or cellulases appeared to affect *P. ultimum*, because of the lack of chitin in its cell walls (Ciopraga et al. 1999). Hence, overproduction of an inducible extracellular protease in *T. harzianum* reduced the growth rate of the fungus (Flores et al. 1997).

The main effect of antifungal compounds on pathogens is the inhibition of spore germination and hyphal elongation. This inhibition was observable only from the morphology, and the detail of the biochemical reactions involved remains unclear. Many works have indicated that the antifungal property of microorganisms follows from the activity of hydrolytic enzymes such as chitinase, which is generated by them. For instance, endochitinase and chitobiosidase generated by *Trichoderma harzianum* inhibited the growth of *Botrytis cinere* and *F. solani* (Lorito et al. 1993). Chitinases produced by *Monascus purpureus* CCRC31499 (Wang et al. 2002a) and *Bacillus amyloliquefaciens* V656 (Wang et al.

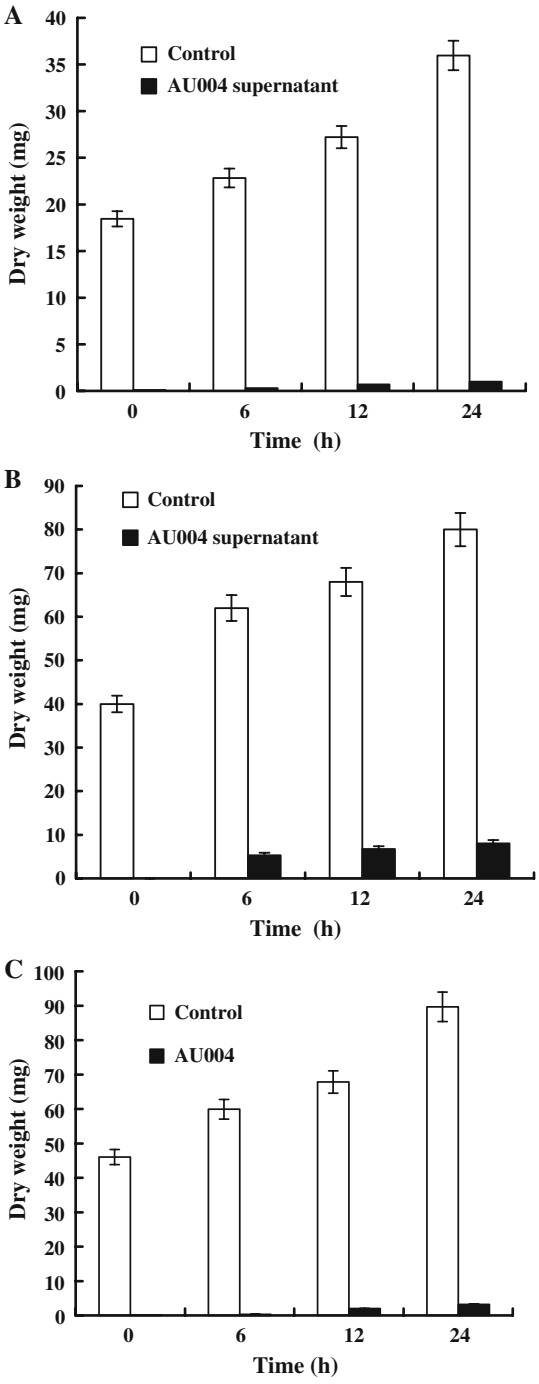

Fig. 2 Effect of *B. cereus* AU004 culture supernatant on biomass production by *F. oxysporum* (**a**), *F. solani* (**b**) and *P. ultimum* (**c**). The pathogenic fungus was grown for 0, 6, 12, and 24 h and the dry weight (mg) recorded by causing the cultures to pass through pre-weighed Whatman No. 1 filter paper

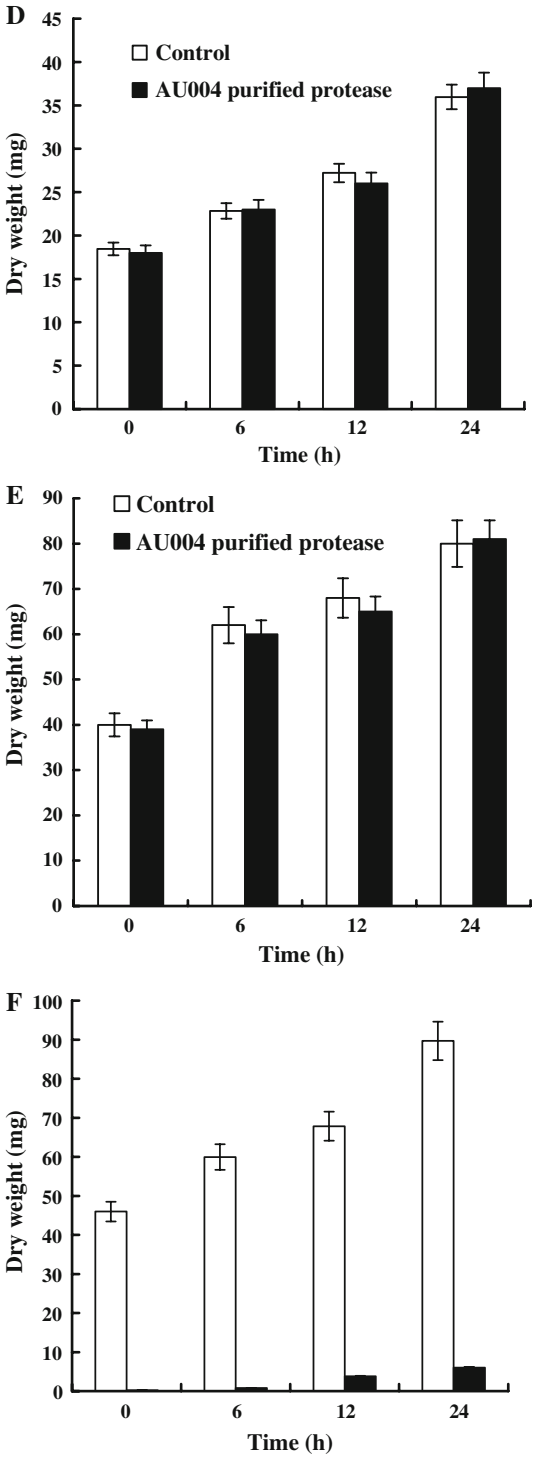

Fig. 3 Effect of *B. cereus* AU004 purified protease on biomass production of *F. oxysporum* (**d**), *F. solani* (**e**) and *P. ultimum* (**f**). The pathogenic fungus was grown for 0, 6, 12, and 24 h and the dry weight (mg) was recorded by allowing the cultures to pass through pre-weighed Whatman No. 1 filter paper

2002b) inhibited *Fusarium oxysporum*. Chitinolytic enzymes produced by *Enterobacter agglomerans* inhibited *Rhizoctonia solani* (Chernin et al. 1995). An inducible extracellular serine protease produced by *Stenotrophomonas maltophilia* strain W81 had the biological control effect of dissolving the mycelium of *P. ultimum* (Dunne et al. 2000).

The antifungal mechanism of *B. cereus* AU004 protease was studied here. Following incubation, almost none of the spores of *P. ultimum* germinated; neither mycelium

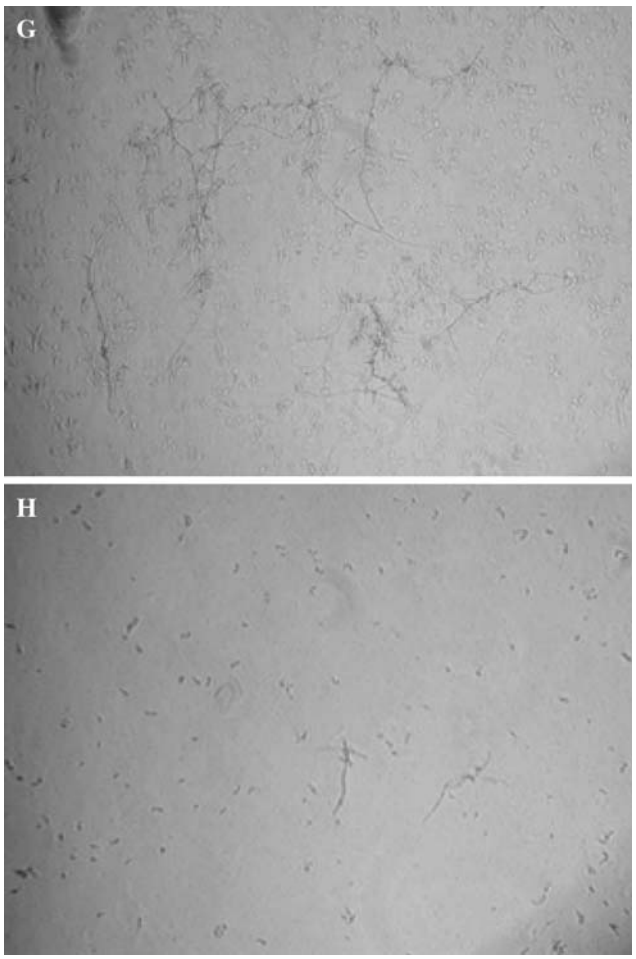

Fig. 4 Effect of antifungal protease from *B. cereus* AU004 on germination of *P. ultimum* spores. **g** Normal germination of *P. ultimum* spores. **h** Inhibition of *P. ultimum* spore germination in presence of AU004 purified protease

cytolysis nor tail-end expansion was observed. This inhibitory phenomenon differed from those associated with the activity of chitinolytic enzymes (Lorito et al. 1993; Mahadevan and Crawford 1997; Gao et al. 2008) and other non-enzymatic compounds (He et al. 1994; Bagnasco et al. 1998; Ciopraga et al. 1999; Zhang et al. 2008). Based on these observations, the antifungal activity of *B. cereus* AU004 may be associated with compounds that are not chitinases. The 28.8 kDa purified protease can inhibit both spore formation and the hyphal germination of the phytopathogen *P. ultimum* in vitro, revealing that this enzyme is involved in the antagonistic interaction between *B. cereus* AU004 and the phytopathogen. This work is the first to describe the isolation of a protease from *Bacillus* spp. that is active against fungi.

Acknowledgements This work was partially supported by a grant from the National Science Council, Taiwan (NSC95-2221-E-272-002). Ted Knoy is appreciated for his editorial assistance.

References

Bagnasco P, De La Fuente L, Gualtieri G, Noya F, Arias A (1998) Fluorescent *Pseudomonas* spp. as biocontrol agents against forage legume root pathogenic fungi. Soil Biol Biochem 30:1317–1322

Chang WT, Chen YC, Jao CL (2007) Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Biores Technol 98:1224–1230

Chernin L, Ismailov A, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl Environ Microbiol 61:1720–1726

Ciopraga J, Gozia O, Tudor R, Brezuica L, Doyle RJ (1999) *Fusarium* sp. growth inhibition by wheat germ agglutinin. Biochim Biophys Acta 1428:424–432

Dunne C, Moenne-Loccoz Y, de Bruijin FJ, O'Gara F (2000) Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotromonas maltophilia* strain W81. Microbiol 146:2069–2078

Flores A, Chet I, Herrera-Estrella A (1997) Improved biocontrol activity of *Trichoderma harzianum* by overexpression of the proteinase-encoding gene prb1. Curr Genet 31:30–37

Gao XA, Ju WT, Jung WJ, Park RD (2008) Purification and characterization of chitosanase from *Bacillus cereus* D-11. Carbohydr Polym 72:513–520

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Peterbauer C, Tronsmo A (1993) Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. Phytopathol 83:313–318

He H, Silo-Suh LA, Handelsman J, Clardy J (1994) Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. Tetrahedron Lett 35:2499–2502

Imoto T, Yagishita K (1971) A simple activity measurement by lysozyme. Agric Biol Chem 35:1154–1156

Janice LM, Carlos RF (2002) Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches' broom disease. BMC Biochem 3:3

Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. The Williams and Wilkins Co, Baltimore

Leelasuphakul W, Sivanunsakul P, Phongpaichit S (2006) Purification, characterization and synergistic activity of β-1, 3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. Enzyme Microb Technol 38:990–997

Lim SH, Kim YS, Kim SD (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. Appl Environ Microbiol 57:510–516

Lindow SE, Arny DC, Upper CD (1983) Biological control of frost injury: establishment and effects of an isolate of *Erwinia herbicola* antagonistic to ice nucleation active bacteria on corn in the field. Phytopathol 73:1102–1106

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A (1993) Chitinolytic enzyme produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathol 83:302–307

Mahadevan B, Crawford DL (1997) Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC 108. Enzyme Microb Technol 20:489–493

Moenne-Loccoz Y, Powell J, Higgins P, McCarthy J, O'Gara F (1998) An investigation of the impact of biocontrol *Pseudomonas fluorescens* F113 on the growth of sugarbeet and the performance of subsequent clover-*Rhizobium* symbiosis. Appl Soil Ecol 7:225–237

Natsch A, Keel C, Hebecker N, Laasik E, Defago G (1998) Impact of *Pseudomonas fluorescens* strain CHA0 and a derivative with improved biocontrol activity on the culturable resident bacterial community on cucumber. FEMS Microbiol Ecol 27:365–380

Scher FM, Baker R (1982) Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. Phytopathol 72:1567–1573

Thomashow LS, Weller DM (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminus* var. *tritici*. J Bacteriol 170:3499–3508

Wang SL, Chang WT (1997) Purification and characterization of two bifunctional chitinase/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Appl Environ Microbiol 63:380–386

Wang SL, Yieh TC, Shih IL (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

Wang SL, Yen YH, Tsiao WJ, Chang WT, Wang CL (2002a) Production of antifungal compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme Microb Technol 31:337–344

Wang SL, Shih IL, Wang CH, Chang WT, Tu YG, Ro JJ, Wang CL (2002b) Production of antifungal compounds from chitin by *Bacillus subtilis*. Enzyme Microb Technol 31:321–328

Zhang B, Xie C, Yang X (2008) A novel small antifungal peptide from *Bacillus* strain B-TL2 isolated from tobacco stems. Peptides 29:350–355