Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 39 (2006) 311–317
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

An antifungal protease produced by *Pseudomonas aeruginosa* M-1001 with shrimp and crab shell powder as a carbon source

Yue-Horng Yen [a], Pei-Ling Li [a], Chuan-Lu Wang [b], San-Lang Wang [c,*]

[a] *Department of Bioindustry Technology, Da-Yeh University, Chang-Hwa 515, Taiwan*
[b] *Department of Environmental Engineering, Lan-Yang Institute of Technology, I-Lan 261, Taiwan*
[c] *Graduate Institute of Life Sciences, Tamkang University, 151 Ying-Chuan Road, Tamsui 251, Taiwan*

Received 27 August 2003; received in revised form 10 May 2005; accepted 7 November 2005

Abstract

Pseudomonas aeruginosa M-1001 produced a protease when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine wastes. An antifungal protease was purified from the culture supernatant to homology. The protease had a molecular weight of 38,000 and a p*I* of 5.7. The optimum pH, optimum temperature, and pH stability of the protease were pH 7, 37 °C, and pH 5–8, respectively. Antifungal activity of the protease was found when using assay based upon inhibition of spores germination and hyphal extension of the fungal *Fusarium solani*.

Keywords: *Pseudomonas aeruginosa*; Protease; Shrimp and crab shell powder; Chitin; Antifungal

1. Introduction

Biological control, or the use of microorganisms or their secretions to prevent plant diseases, offers an attractive alternative or supplement for the control of plant diseases without the negative impact of chemical control. Therefore, biological control tactics have become an important approach to facilitating sustainable agriculture [1–6]. Chitin bioconversion has been proposed as a waste treatment alternative to the disposal of shellfish waste [7–9]. To further enhance the utilization of chitin-containing marine crustacean waste, we have recently investigated the bioconversion of shrimp and crab shell powder (SCSP) for bio-fungicide production [10–14]. Such as *Pseudomonas aeruginosa* K-187 [6,7,10], *Bacillus subtilis* W113 [11], *B. subtilis* W118 [11], *B. amyloliquefaciens* V656 [12], *Monascus purpureus* CCRC31499 [13,14]. *P. aeruginosa* M-1001, lysozyme inhibitor and lytic enzyme producing strain, was isolated from the soil of Taiwan [15,16]. In the present work, we further found that *P. aeruginosa* M-1001 displayed antifungal and protease activities when cultured in an SCSP medium. The effects of the antifungal compounds on the spore germination and germ spore elongation of pathogenic *Fusarium solani* were investigated. The purification and characterization of the antifungal protease thus produced were also investigated.

2. Materials and methods

2.1. Materials

The SCSP used in these experiments was prepared as described earlier [12]. In the preparation of the SCSP, the shrimp and crab shells collected from a marine food processing industry were washed thoroughly with tap water and then steamed. The solid material obtained was dried, milled, and sieved to powder with diameters <0.053 mm. Chitin flake and chitosan powder from crab shell were purchased from Biotech Co. (Kau-shyuon, Taiwan). Powdered chitin, ethylene glycol chitin, and lyophilized cells of *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co., St. Louis, MO. Colloidal chitin was prepared from powdered chitin according to the method of Jeniaux [17]. Cell suspensions of *M. lysodeikticus* were prepared as described previously [10]. All other reagents used were of the highest grade available.

2.2. Effect of culture conditions

P. aeruginosa M-1001 was isolated from the soil in Taiwan [15,16] and maintained on nutrient agar plates at 37 °C. In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7), and gradually supplemented with the various carbon sources to be investigated. The major carbon sources being investigated included SCSP, chitin, chitosan, Chinese herb, tea lees, and cellulose. They were added and investigated in one kind at a time fashion. One hundred milliliter of

* Corresponding author. Tel.: +886 2 2626 9425/2809 6078; fax: +886 2 8631 1015/2809 1892.
E-mail address: sabulo@mail.tku.edu.tw (S.-L. Wang).

0141-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.11.050

the resultant medium in a 250 mL Erlenmeyer flask was aerobically cultured at 30 °C for 24 h on a rotary shaker (150 rpm). After centrifugation (8000 × *g*, 4 °C, for 20 min, Beckman J2-21 M/E), the supernatant was used for bioassay. Usually an effective experimental prior condition was used as the basis for the later experiment until the optimal culture condition was obtained. With the use of the optimal culture composition, the effects of the initial pH, temperature, culture volume, and cultivation time on the production of antifungal compounds were investigated in the same fashion until the optimum culture condition was found.

2.3. Preparation of crude antifungal compounds

P. aeruginosa M-1001 was cultured under optimal culture condition. After centrifugation (8000 × *g*) at 4 °C for 20 min, the supernatant was subjected to ammonium sulfate precipitation. The resultant precipitate was collected and dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), followed by dialysis against the same buffer overnight. The resultant dialyzate was filtered aseptically through 0.45 μm-pore-size membrane filters and used for the bioasssays.

2.4. Antifungal action of the antifungal compounds

The antifungal activity for the antifungal compounds was estimated using a growth inhibition assay described earlier [1]. The test fungus, *F. solani*, was kindly supplied by Dr. Chaur-Tseuen Lo, Department of Plant Pathology, Taiwan Agricultural Research Institute, Taichung, Taiwan. Fungal spores of *F. solani* were grown on petri plates filled with potato dextrose agar (PDA). After 10 days of incubation at 25 °C, the fungal colonies were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through sterilized gauges. The filtrate was adjusted with sterile water to a concentration of 1×10^6 spores per mL, and stored at 4 °C. To test the antagonistic effect of the antifungal compounds produced by *P. aeruginosa* M-1001, Petri plates were filled with molten PDA pre-cooled to 45 °C, and divided into two groups (triplicate for each). To each plate in the experimental group (*E*) an appropriate amount of the antifungal compounds was added. To those of the control group (*C*), an equal amount of sterile buffer was added. After the plates were cooled, the fungal inoculum was then placed onto the 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. If the inhibitory ratio was greater than 20%, the test strain would be considered inhibited and the minimal inhibitory concentration (MIC) for that strain was then determined:

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

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.

2.5. Measurement of enzyme activity

For measuring protease activity, a diluted enzyme solution (0.2 mL) was mixed with 2.5 mL of 1% casein in phosphate buffer pH 7 and incubated for 10 min at 37 °C. The reaction was terminated by adding 5 mL of 0.19 M trichloroacetic acid (TCA). The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured by the method of Todd with tyrosine as the reference compound [18].

Chitinase activity was measured with colloidal chitin as a substrate [10]. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita [19] with *N*-acetylglucosamine as a reference compound. The activity of ethylene glycol chitinase and lysozyme were assayed by the procedures as described previously [10].

2.6. Determination of molecular weight and isoelectric point

The molecular weight of the purified protease was determined by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS–PAGE) according to the method of Weber and Osborn [20]. The standard proteins used for calibration were phosphorylase b (molecular weight = 97,000), albumin (67,000), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (20,100), and α-lactabumin (14,400).

Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing 2-mercatpethanol. 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.

The isoelectric point of the purified protease was estimated by chromatofocusing. The protease soultion (1 mL) was loaded onto a chromatofocusing PBE 94 column (0.9 by 27 cm) equilibrated with 50 mM Tris–HCl buffer (pH 6), and the elution was done with Polybufer 74-Tris–HCl (pH 6) as described in the manufacturer's manual (Pharmicia).

2.7. Purification of the protease

(i) *Production of protease*: For the production of protease, *P. aeruginosa* M-1001 was grown in 75 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SCSP, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ (pH 8). Two milliliters of the seed culture was transferred into 75 mL of the same medium and grown in an orbital shaking incubator for 36 h at 37 °C and pH 8. The culture broth was centrifuged (4 °C and 12,000 × *g* for 20 min), and the supernatant was used for further purification by chromatography.

(ii) *DEAE-Sepharose CL-6B chromatography*: To the cell-free culture broth (1800 mL), ammonium sulfate was added (608 g/L). The resultant mixture was kept at 4 °C overnight and the precipitate formed was collected by centrifugation at 4 °C for 20 min at 12,600 × *g*. The precipitate was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and dialyzed against the buffer. The resultant dialysate (100 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 by 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 7). The unadsorbed materials were washed from the column with the same eluting buffer, and the enzymes were fractionated with a linear gradient of 0–1 M NaCl in 50 mM phosphate buffer. The flow rate was 75 mL/h. The eluted fractions were dialyzed against 50 mM sodium phosphate buffer (pH 7, 4 °C) for 24 h to remove NaCl, and assayed for antifungal and protease activities.

2.8. Protein determination

Protein was determined by the method of Lowry et al. [21] with bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

2.9. pH and thermal stability

The pH stability of the samples was determined by measuring the residual inhibitory activity at pH 7 as described above after dialyzing the samples against a 50 mM buffer solution of various pHs (pH 3–11) in seamless cellulose tubing (Sankyo, Japan). The buffer systems used were glycine–HCl (50 mM, pH 3), acetate (50 mM, pH 4.5), phosphate (50 mM, pH 6–8), Na_2CO_3–$NaHCO_3$ (50 mM, pH 9–11). The thermal stability of the samples was studied by heating the samples at 100 °C for various time periods. The residual activity was measured as described above (using *F. solani* as target).

2.10. Spore germination affected by the antifungal protease

An amount of 0.25 mL of fungal spores (10^6/mL) of *F. oxysporum*, 0.5 mL of potato dextrose broth, and 0.25 mL of antifungal protease (5 mg) was added into the eppendorf tube. The resultant mixture was incubated at 25 °C, and the spores were observed by using a light microscope at various intervals.

3. Results

3.1. Effect of culture conditions

To study the effect of carbon sources on the production of antifungal compounds, growth was carried out in basal medium as